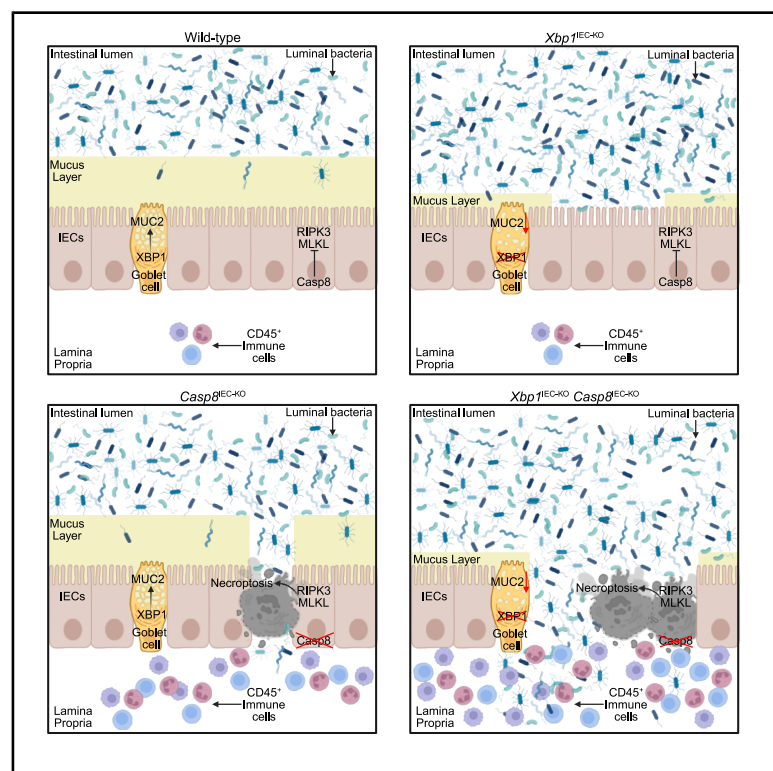


# Unfolded protein response transcription factor XBP1 suppresses necroptosis-induced colitis by reinforcing the mucus barrier

## Graphical abstract



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## In brief

Inflammatory bowel disease is a multifactorial disease driven by combinations of susceptibility factors. Kaya et al. demonstrate how XBP1 deficiency impairs MUC2-mediated mucus barrier formation. Diminished mucus formation synergizes with epithelial cell necroptosis to exacerbate colitis. This work highlights how combined defects in epithelial integrity and the mucus layer drive chronic colon inflammation.

## Highlights

- XBP1 deficiency exacerbates colitis development in *Casp8<sup>IEC-KO</sup>* and *Fadd<sup>IEC-KO</sup>* mice
- XBP1 controls colonic mucus barrier integrity by regulating MUC2 expression
- Mucus layer impairment synergizes with epithelial necroptosis to cause severe colitis
- ER stress and necroptosis synergize to drive severe colon inflammation



Article

# Unfolded protein response transcription factor XBP1 suppresses necroptosis-induced colitis by reinforcing the mucus barrier

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## SUMMARY

Endoplasmic reticulum (ER) stress and necroptosis are associated with the pathogenesis of inflammatory bowel disease (IBD); however, the potential crosstalk between these pathways is unclear. Here, we show that intestinal epithelial cell (IEC)-specific X-box binding protein 1 (XBP1) deficiency strongly aggravates the development of necroptosis-induced colitis, but not ileitis, in mice lacking caspase-8 or its adapter Fas associated with death domain (FADD) in IECs. Mechanistically, XBP1 ablation led to diminished mucin 2 (MUC2) expression and impaired mucus layer formation in the colon, which allowed bacteria to penetrate and reach the epithelial surface. This was not sufficient to trigger colitis in the presence of an intact epithelial monolayer but synergized with IEC necroptosis to induce severe colon inflammation. Our results revealed that XBP1 and caspase-8 control different components of the intestinal barrier that synergize to maintain mucosal immune homeostasis and prevent colon inflammation. This could be relevant for the better understanding of the mechanisms causing IBD.

## INTRODUCTION

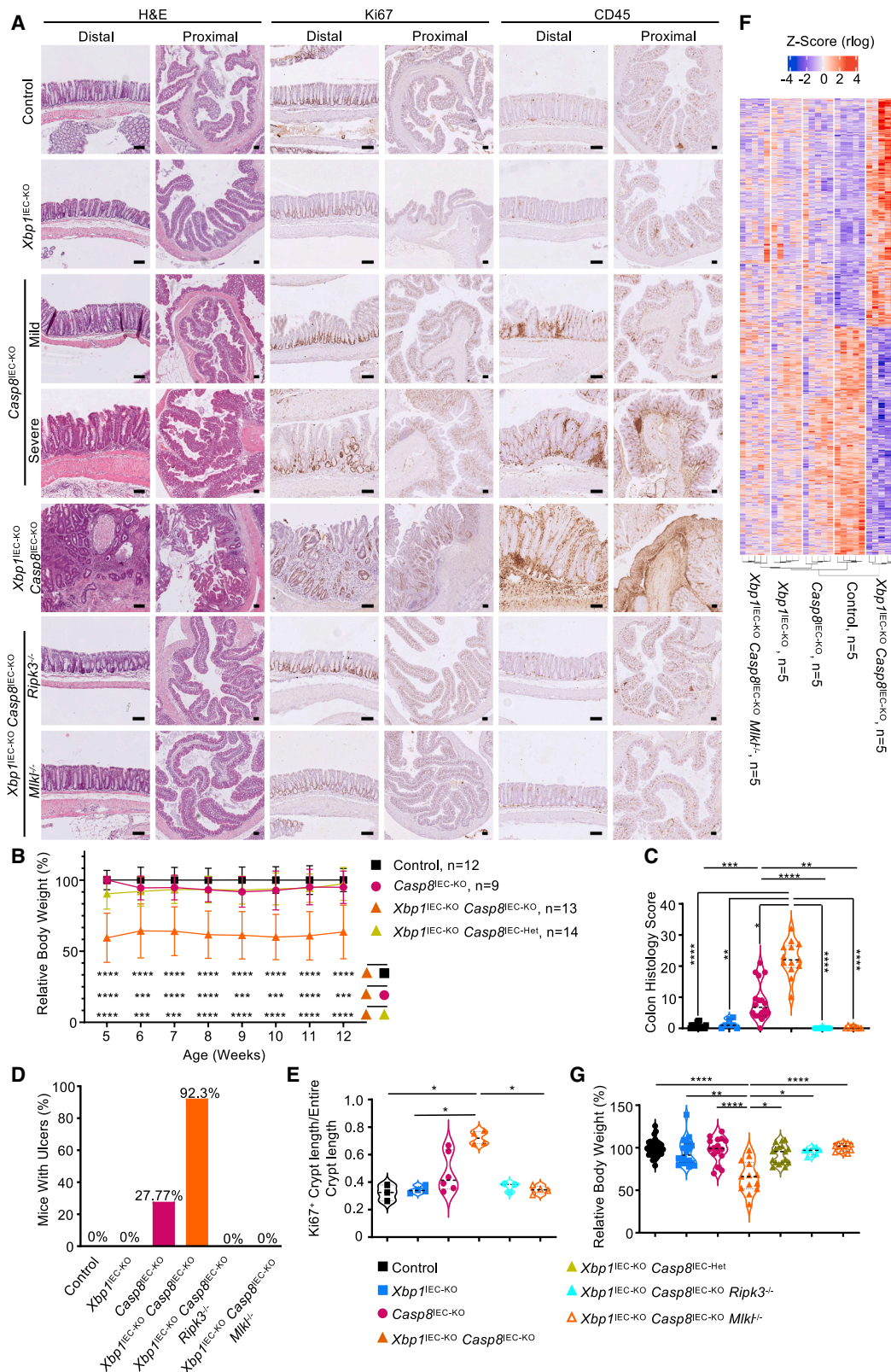
Intestinal epithelial cells (IECs) form a polarized monolayer that plays a crucial role in the maintenance of mucosal immune homeostasis by providing a physical and biochemical barrier separating the lamina propria from the luminal microbiota.<sup>1–3</sup> A delicate balance between proliferation, differentiation, and death of IECs is critical for the healthy functioning of the intestinal epithelial barrier.<sup>4–6</sup> Perturbation of the intestinal barrier is linked to the pathogenesis of inflammatory bowel diseases (IBDs) such as Crohn's disease (CD) and ulcerative colitis (UC).<sup>7</sup> Goblet cells are specialized secretory epithelial cells that produce and secrete glycosylated, hydrophilic, polymer-forming proteins called mucins. These mucins form a mucus layer that covers the intestinal epithelium and is important for maintaining an effective barrier.<sup>8,9</sup> The colon harbors two distinct layers of mucus: (1) an inner stratified mucus layer that is firmly attached to the colonic epithelium and prevents bacteria from contacting the colonic epithelium and (2) an outer mucus layer that is permeable to bacteria and easily removed.<sup>10,11</sup>

In patients suffering from UC, colonic biopsies show that the inner mucus barrier is thinner, loosely attached to the epithelium, and penetrable to bacteria,<sup>12</sup> suggesting that impaired mucus layer formation could contribute to the pathology.

Increased intestinal permeability is seen in asymptomatic relatives of IBD patients, indicating that genetic factors affecting the intestinal barrier likely contribute to disease susceptibility.<sup>13–18</sup> Intestinal biopsies from IBD patients also suggest elevated levels of IEC death, which likely contribute to intestinal inflammation by compromising the intestinal epithelial barrier.<sup>19–23</sup> In addition, patients with mutations affecting proteins regulating cell death, such as receptor-interacting protein kinase 1 (RIPK1)<sup>24,25</sup> and caspase-8,<sup>23</sup> show intestinal inflammation. Studies in genetic mouse models provide experimental evidence that death of IECs drives intestinal inflammation.<sup>26–31</sup> Mice lacking caspase-8 or its adapter Fas associated with death domain (FADD), specifically in IECs (*Casp8*<sup>IEC-KO</sup> and *Fadd*<sup>IEC-KO</sup>, respectively), develop spontaneous intestinal inflammation in both the colon and small intestine. This is triggered by necroptosis of







**Figure 1. IEC-specific XBP1 ablation exaggerates necroptosis-induced colitis in *Casp8*<sup>IEC-KO</sup> mice**

(A) Representative images of Swiss-roll colon sections from 8- to 12-week-old mice with the indicated genotypes stained with H&E or immunostained for CD45 and Ki67. Scale bars, 100  $\mu$ m.

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epithelial cells mediated by RIPK3 and its substrate, mixed-lineage kinase-like (MLKL).<sup>32–35</sup> Therefore, death of IECs triggers intestinal inflammation and could contribute to IBD pathogenesis.

Whereas the etiology of IBD remains poorly understood, genetic factors and environmental cues such as the intestinal microbiota can contribute to the initiation and chronicity of intestinal inflammation.<sup>5,36,37</sup> Genome-wide association studies (GWASs) identified more than 200 IBD susceptibility loci.<sup>38–40</sup> One of the genes associated with both CD and UC encodes X-box binding protein 1 (XBP1).<sup>41</sup> XBP1 is a transcription factor that plays a crucial role in the unfolded protein response (UPR) by regulating genes involved in protein folding, secretion, and degradation to maintain cellular homeostasis.<sup>42</sup> IEC-specific deletion of XBP1 in mice causes unresolved endoplasmic reticulum (ER) stress, resulting in loss of paneth cells, spontaneous small intestinal inflammation, and increased susceptibility to dextran sulfate sodium (DSS)-induced colitis.<sup>41</sup>

IBD is a multifactorial disease precipitated by a combination of genetic and environmental factors.<sup>5,36,37</sup> However, how the crosstalk of different genes and pathways contributes to intestinal inflammation remains largely unresolved. Here, we show that XBP1 ablation in IECs exaggerated colitis development in *Casp8*<sup>IEC-KO</sup> and *Fadd*<sup>IEC-KO</sup> mice, revealing that deregulated ER stress responses synergize with epithelial cell necroptosis to trigger severe intestinal inflammation. Mechanistically, XBP1 was required for expression of mucin 2 (MUC2) and the formation of a functional mucus layer. This limited bacterial invasion to the lamina propria when the intestinal barrier was breached by IEC necroptosis. These results suggest that combined defects compromising mucus formation and epithelial cell survival contribute to the pathogenesis of IBD.

## RESULTS

### XBP1 suppresses necroptosis-induced colitis development in *Casp8*<sup>IEC-KO</sup> and *Fadd*<sup>IEC-KO</sup> mice

To study the role of XBP1 and caspase-8 in IECs, we generated *Xbp1*<sup>fl/fl</sup> × *Vil1-Cre* (*Xbp1*<sup>IEC-KO</sup>) and *Casp8*<sup>fl/fl</sup> × *Vil1-Cre* (*Casp8*<sup>IEC-KO</sup>) mice. In line with previous reports,<sup>35,41,43</sup> *Xbp1*<sup>IEC-KO</sup> mice did not develop spontaneous pathology in the colon, whereas *Casp8*<sup>IEC-KO</sup> mice displayed mild-to-severe colitis characterized by hyperplasia, mild epithelial erosion, dying IECs, and infiltration of immune cells (Figure 1A). To assess the potential crosstalk between XBP1 and caspase-8 in regulating intestinal epithelial homeostasis, we generated and analyzed *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> mice, which lack both XBP1 and caspase-8 specifically in IECs. *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> mice showed markedly

reduced body weight compared with *Casp8*<sup>IEC-KO</sup> and *Xbp1*<sup>fl/fl</sup> × *Casp8*<sup>fl/fl</sup> × *Villin-cre*<sup>tg/WT</sup> (*Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-Het</sup>) mice (Figure 1B), indicating that combined loss of XBP1 and caspase-8 in IECs caused severe intestinal pathology. Indeed, histological analysis of colon tissue sections revealed that *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> mice developed severe colitis, manifesting with epithelial hyperproliferation, increased numbers of dying IECs, extensive epithelial erosion leading to the formation of ulcers scattered throughout the entire colon, increased influx of immune cells in mucosa and submucosa, and the presence of extensive crypt abscesses (Figures 1A and S1A). Quantification of histological colitis score, ulcer formation, and epithelial cell proliferation confirmed that *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> mice developed exacerbated colon pathology compared with *Casp8*<sup>IEC-KO</sup> mice (Figures 1C–1E). Moreover, RNA sequencing (RNA-seq) revealed increased expression of inflammatory genes in the colons of *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> compared with *Xbp1*<sup>IEC-KO</sup> and *Casp8*<sup>IEC-KO</sup> mice, further supporting that combined loss of XBP1 and caspase-8 caused more severe colon inflammation compared with deficiency of either gene alone (Figures 1F and S1B; Table S1). We showed previously that inhibition of necroptosis by genetic deficiency of RIPK3 or MLKL prevents colitis development in *Casp8*<sup>IEC-KO</sup> mice.<sup>35</sup> We therefore asked whether necroptosis also contributes to the exacerbated colitis observed upon combined inhibition of XBP1-mediated UPR and caspase-8 signaling. To address this question, we generated and analyzed *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> *Ripk3*<sup>−/−</sup> and *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> *MLK*<sup>−/−</sup> mice. Macroscopic, immunohistological, and RNA-seq analyses showed that inhibition of RIPK3-MLKL-mediated necroptosis fully restored the body weight of, and prevented the development of colitis in, *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> mice (Figures 1A and 1C–1G; Table S1). Mice with IEC-specific deletion of FADD develop spontaneous colitis that is more severe than in *Casp8*<sup>IEC-KO</sup> mice.<sup>32,35</sup> Consistent with our findings in *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> mice, side-by-side immunohistological assessment and quantification demonstrated that *Xbp1*<sup>IEC-KO</sup> *Fadd*<sup>IEC-KO</sup> mice developed more severe colitis compared with *Fadd*<sup>IEC-KO</sup> mice (Figures S1C–S1F). RIPK3 deficiency also prevented the development of colitis in *Xbp1*<sup>IEC-KO</sup> *Fadd*<sup>IEC-KO</sup> *Ripk3*<sup>−/−</sup> mice (Figures S1C–S1F). Taken together, these results revealed that XBP1 acts in epithelial cells to suppress necroptosis-induced inflammatory colitis in *Fadd*<sup>IEC-KO</sup> and *Casp8*<sup>IEC-KO</sup> mice.

### XBP1 deficiency did not exacerbate ileitis in *Casp8*<sup>IEC-KO</sup> and *Fadd*<sup>IEC-KO</sup> mice

*Xbp1*<sup>IEC-KO</sup> mice develop spontaneous ileitis, manifesting with epithelial hyperplasia, loss of paneth cells, and infiltration of

(B) Graph depicting the relative body weight of mice with the indicated genotypes compared with their littermate controls between 5 and 12 weeks of age. Graph shows mean ± SEM. *p* values were calculated by two-way ANOVA. \*\*\**p* < 0.005, \*\*\*\**p* < 0.0001.

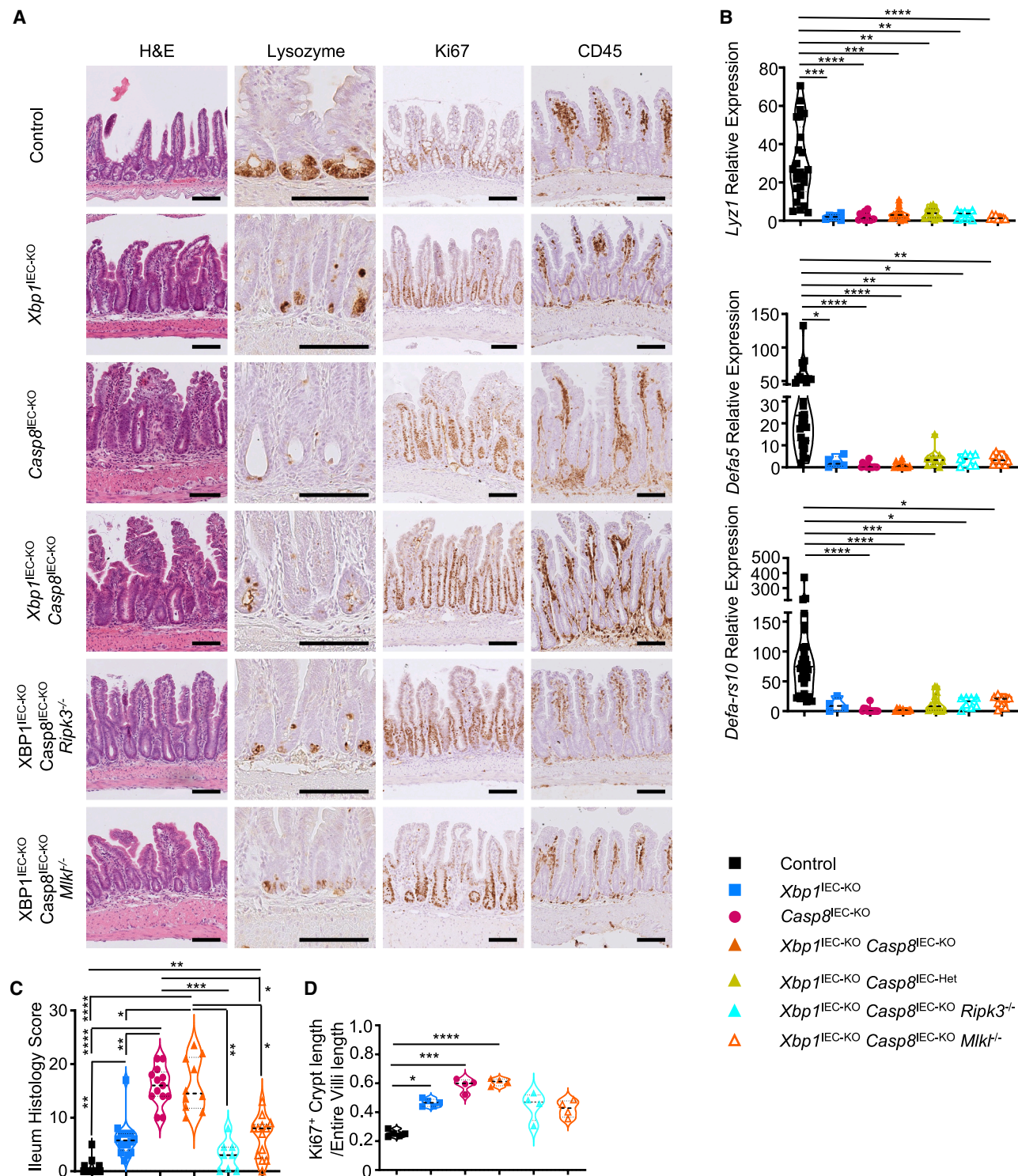
(C–E) Graphs depicting colon histopathology scores (C), the percentage of mice bearing ulcers (D), and the length of Ki67-positive crypt per entire crypt (E) of 8- to 12-week-old mice with the indicated genotypes. *p* values were calculated by non-parametric ANOVA. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.005, \*\*\*\**p* < 0.0001. Each dot represents one mouse.

(F) Heatmap depicting RNA-seq gene expression data from the distal colon of 8- to 12-week-old mice with the indicated genotypes (*n* = 5/genotype). The heatmap includes genes differentially regulated in *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> mice compared with wild-type controls (shrunk fold change of ≥1.5 or ≤1.5, adjusted *p* value of ≤0.05). Columns represent individual mice, hierarchically clustered according to differential gene expression.

(G) Graphs depicting relative body weight of 8- to 12-week-old mice with the indicated genotypes normalized to age- and sex-matched littermate controls. Violin plots show the median of biological replicates and the interquartile range of data. *p* values were calculated by non-parametric ANOVA. \**p* < 0.05, \*\**p* < 0.01, \*\*\*\**p* < 0.0001. Each dot represents one mouse.

Sample sizes are described in Table S2.





**Figure 2. Inhibition of caspase-8-mediated apoptosis and RIPK3-MLKL-induced necroptosis does not prevent paneth cell loss induced by XBP1 deficiency in IECs**

(A) Representative images of Swiss-roll ileal sections from 8- to 12-week-old mice with the indicated genotypes stained with H&E or immunostained for lysozyme and CD45 and Ki67. Scale bars, 100  $\mu$ m.

(B) Graphs depicting relative mRNA expression of *Lyz1*, *Defa5*, and *Defa-rs10* measured by RT-qPCR in the ilea of 8- to 12-week-old mice with indicated genotypes.

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immune cells in the mucosa.<sup>41,44</sup> Moreover, *Casp8*<sup>IEC-KO</sup> mice develop spontaneous ileitis that is dependent on RIPK3-MLKL-mediated necroptosis.<sup>35,45</sup> To assess whether combined XBP1 and caspase-8 deficiency could also aggravate ileitis, we compared the ileal pathology of *Xbp1*<sup>IEC-KO</sup>, *Casp8*<sup>IEC-KO</sup>, and *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> mice. Immunostaining of ileal sections from *Xbp1*<sup>IEC-KO</sup> mice for lysozyme revealed reduced numbers of paneth cells in crypts, which correlated with diminished mRNA expression of paneth-cell-specific genes, including lysozyme (*Lyz1*), *Defa5*, and *Defa-rs10* (Figures 2A and 2B). However, we did not observe markedly increased infiltration of CD45<sup>+</sup> immune cells in the ileum of *Xbp1*<sup>IEC-KO</sup> mice, which was reported in previous studies,<sup>41,44</sup> suggesting that differences in the genetic background and/or the microbiota might influence the small intestinal pathology of these animals (Figure 2A). In line with previous reports, *Casp8*<sup>IEC-KO</sup> mice developed spontaneous ileitis, manifesting with a nearly complete loss of paneth cells, epithelial hyperplasia, presence of dying IECs, and increased immune cell infiltration (Figures 2A and 2B). *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> mice also developed spontaneous ileitis, characterized by epithelial hyperplasia, loss of paneth cells, dying IECs, and infiltration of immune cells in the lamina propria (Figures 2A–2D). Side-by-side histopathological and gene expression analysis showed that the small intestinal pathology observed in *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> mice was very similar to the ileitis observed in *Casp8*<sup>IEC-KO</sup> mice (Figures 2A–2D). Although a significant increase in the ileum histology score was observed in the *Xbp1*<sup>IEC-KO</sup> mice (Figure 2C), this was mainly due to the crypt hyperplasia associated with this mouse model (Figure 2D). Furthermore, *Xbp1*<sup>IEC-KO</sup> *Fadd*<sup>IEC-KO</sup> mice also developed a small intestinal pathology that was very similar to the ileitis observed in *Fadd*<sup>IEC-KO</sup> mice (Figures S2A–S2C). Taken together, these results demonstrated that XBP1 ablation in IECs did not alter the severity of ileitis in *Casp8*<sup>IEC-KO</sup> and *Fadd*<sup>IEC-KO</sup> mice, in stark contrast to the colon, where XBP1 deficiency aggravated necroptosis-induced inflammation.

### Combined inhibition of apoptosis and necroptosis did not prevent paneth cell loss in *Xbp1*<sup>IEC-KO</sup> mice

IEC apoptosis is proposed to play a role in the pathology developing in the ileum of *Xbp1*<sup>IEC-KO</sup> mice.<sup>41</sup> To address whether caspase-8-mediated apoptosis and RIPK3-MLKL-induced necroptosis contribute to the epithelial hyperplasia and paneth cell loss in *Xbp1*<sup>IEC-KO</sup> mice, we examined small intestinal tissues from *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> *Ripk3*<sup>−/−</sup>, *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> *Mkl1*<sup>−/−</sup>, and *Xbp1*<sup>IEC-KO</sup> *Fadd*<sup>IEC-KO</sup> *Ripk3*<sup>−/−</sup> animals. Immunohistological and gene expression analysis revealed that RIPK3 or MLKL deficiency reduced the severity of ileitis in *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> and *Xbp1*<sup>IEC-KO</sup> *Fadd*<sup>IEC-KO</sup> mice to the levels of the *Xbp1*<sup>IEC-KO</sup> mice (Figures 2A–2D and S2A–S2C). However, immunostaining for Ki67 and lysozyme revealed that combined inhibition of caspase-8-dependent apoptosis and RIPK3-MLKL-dependent necroptosis did not prevent the epithelial hyperplasia and paneth cell loss induced by XBP1 deficiency, with *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> *Ripk3*<sup>−/−</sup>, *Xbp1*<sup>IEC-KO</sup>

*Casp8*<sup>IEC-KO</sup> *Mkl1*<sup>−/−</sup>, and *Xbp1*<sup>IEC-KO</sup> *Fadd*<sup>IEC-KO</sup> *Ripk3*<sup>−/−</sup> mice displaying a similar small intestinal pathology to that in *Xbp1*<sup>IEC-KO</sup> mice (Figures 2A–2D and S2A–S2C). Consistent with the lysozyme immunostainings, quantitative RT-PCR (RT-qPCR) analysis revealed considerably reduced expression of *Lyz1*, *Defa5*, and *Defa-rs10* in *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> *Ripk3*<sup>−/−</sup> and *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> *Mkl1*<sup>−/−</sup> mice (Figure 2B). We wondered whether caspase-8- and necroptosis-independent cell death could contribute to paneth cell loss and epithelial hyperplasia induced by XBP1 deficiency. We therefore stained ileal sections from *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> *Ripk3*<sup>−/−</sup>, *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> *Mkl1*<sup>−/−</sup>, and *Xbp1*<sup>IEC-KO</sup> *Fadd*<sup>IEC-KO</sup> *Ripk3*<sup>−/−</sup> mice for cleaved caspase-3 (CC3) and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL). These experiments did not reveal increased numbers of dying IECs within the crypts of *Xbp1*<sup>IEC-KO</sup>, *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> *Ripk3*<sup>−/−</sup>, and *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> *Mkl1*<sup>−/−</sup> mice compared with control mice (Figure S2D). In line with our previous findings, we detected increased numbers of CC3<sup>+</sup> and TUNEL<sup>+</sup> IECs in the ilea of *Xbp1*<sup>IEC-KO</sup> *Fadd*<sup>IEC-KO</sup> *Ripk3*<sup>−/−</sup> mice compared with control mice (Figure S2D).<sup>35</sup> Collectively, our results showed that FADD-caspase-8-dependent apoptosis and RIPK3-MLKL-mediated necroptosis do not drive paneth cell loss and epithelial hyperplasia in *Xbp1*<sup>IEC-KO</sup> mice.

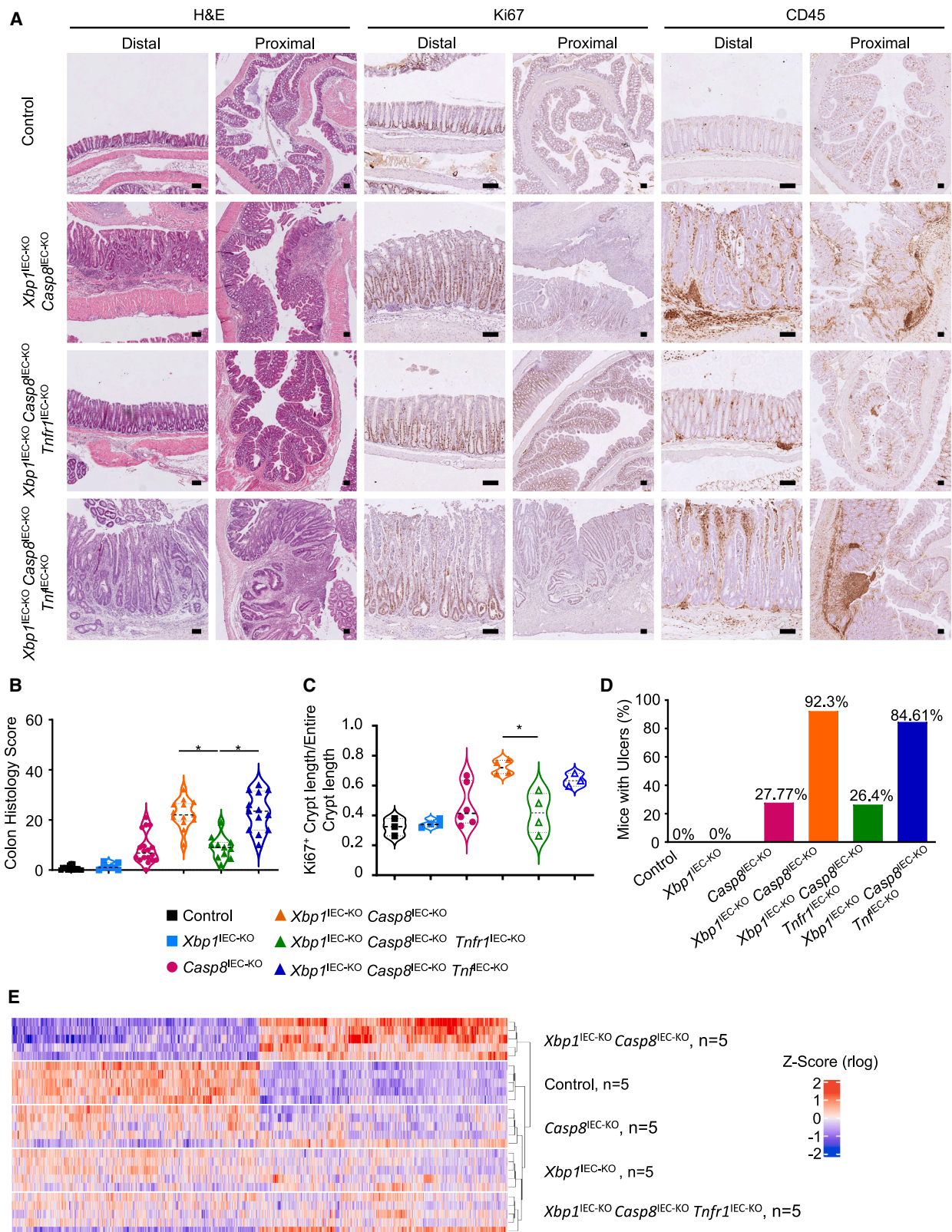
### Epithelial-intrinsic TNFR1 signaling drives colitis exacerbation in *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> mice

IEC-specific tumor necrosis factor receptor 1 (TNFR1) deficiency mitigates colitis development in *Casp8*<sup>IEC-KO</sup> mice.<sup>35</sup> TNFR1 also contributes to ileitis development in *Xbp1*<sup>IEC-KO</sup> mice.<sup>44</sup> To address whether TNFR1 signaling in IECs mediates the severe colitis of *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> mice, we generated and analyzed *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> *Tnfr1*<sup>IEC-KO</sup> mice. Indeed, immunohistological assessment and RNA-seq analysis revealed that epithelial TNFR1 deficiency strongly suppressed colitis development in *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> *Tnfr1*<sup>IEC-KO</sup> mice (Figures 3A–3E). It has been proposed that tumor necrosis factor (TNF) production, potentially driven by nuclear factor  $\kappa$ B (NF- $\kappa$ B) activation in XBP1-deficient IECs and/or paneth cells, could contribute to enteritis in *Xbp1*<sup>IEC-KO</sup> mice.<sup>44</sup> Therefore, we reasoned that elevated levels of IEC-derived TNF might contribute to the exaggerated colitis in *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> mice. To test this hypothesis, we generated and analyzed *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> *Tnfr1*<sup>IEC-KO</sup> mice lacking XBP1, caspase-8, and TNF specifically in IECs. Immunohistological analysis of colon sections demonstrated that *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> *Tnfr1*<sup>IEC-KO</sup> mice developed colitis with similar severity to that observed in *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> mice (Figures 3A–3D), arguing against a role of IEC-derived TNF in driving the pathology. Moreover, IEC-specific deletion of TNF or TNFR1 did not alleviate paneth cell loss and the small intestinal pathology of *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> mice (Figures S3A–S3D). These results are consistent with our previous findings showing that TNFR1 does not play an important role in necroptosis-driven small intestinal

(C and D) Graphs depicting ileal histopathology scores (C) and the length of Ki67-positive crypt per entire villi (D) of 8- to 12-week-old mice from indicated genotypes. Violin plots show the median of biological replicates and the interquartile range of data.

*p* values were calculated by non-parametric ANOVA. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.005, \*\*\*\**p* < 0.0001. Each dot represents one mouse. Sample sizes are indicated in Table S2.





**Figure 3. TNFR1 signaling in IECs aggravates the severity of colitis in *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> mice**

(A) Representative images of Swiss-roll colon sections from 8- to 12-week-old mice with the indicated genotypes stained with H&E or immunostained for Ki67 and CD45. Scale bars, 100  $\mu$ m.

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inflammation in *Casp8*<sup>IEC-KO</sup> mice.<sup>35</sup> Together, these results showed that epithelial-intrinsic TNFR1 signaling drives colitis exacerbation in *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> mice, but TNF derived from IECs does not play an important role.

### IEC-specific XBP1 ablation compromises the formation of the colonic mucus layer

Analysis of the RNA-seq data revealed that the only transcript that was profoundly reduced in the colons of *Xbp1*<sup>IEC-KO</sup> mice was *Muc2* (Figure 4A; Table S1). RT-qPCR analysis, immunostaining, and immunoblotting with specific antibodies confirmed reduced expression of MUC2 mRNA and protein in the colons of *Xbp1*<sup>IEC-KO</sup> compared with control mice (Figures 4B, S4A, and S4B). The number of goblet cells was not reduced in the colons of *Xbp1*<sup>IEC-KO</sup> mice, but they were smaller and exhibited diminished MUC2 expression compared with control mice (Figure S4C). MUC2 is the most abundantly secreted gel-forming mucin in the intestine and a major constituent of the mucus layer.<sup>46,47</sup> Consistently, Alcian blue-PAS staining revealed diminished mucin levels in the colons of *Xbp1*<sup>IEC-KO</sup> mice compared with their littermate controls (Figure S4A). Analysis of the RNA-seq data showed that other mucus-associated genes were not significantly altered in the colons of *Xbp1*<sup>IEC-KO</sup> compared with control mice (Table S1). MUC2 is a crucial constituent of the impenetrable mucus layer that lines the colonic epithelium and prevents luminal bacteria from coming in contact with the enterocytes.<sup>48</sup> We hypothesized that reduced *Muc2* expression caused by IEC-specific XBP1 deficiency could compromise mucus layer formation, which could contribute to the exaggerated colitis development in *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> and *Xbp1*<sup>IEC-KO</sup> *Fadd*<sup>IEC-KO</sup> mice by allowing increased numbers of bacteria to come in contact with the epithelium. To study whether reduced MUC2 production could disrupt the formation of a protective mucus layer in *Xbp1*<sup>IEC-KO</sup> mice, we examined cross-sections from distal colon tissue containing fecal material fixed in Carnoy's Fixative to preserve the mucus structure.<sup>49</sup> Alcian blue-periodic acid-Schiff (PAS) staining as well as MUC2 immunostaining of these sections revealed a well-formed thick mucus layer in colons from control mice (Figure 4C). In stark contrast, the thickness of the mucus layer in the colons of *Xbp1*<sup>IEC-KO</sup> mice was markedly diminished, with certain areas completely lacking a mucus layer (Figure 4C). To quantify and compare the thickness of the mucus layer, the total area of mucus was measured and normalized to the periphery of the colonic epithelium for each mouse. This analysis revealed that IEC-specific XBP1 deletion led to a strong reduction of the overall mucus layer thickness (Figure 4D). To assess whether the separation of bacteria from the colonic epithelial surface was also compromised in the colons of *Xbp1*<sup>IEC-KO</sup> mice, we performed fluorescence *in situ* hybridization (FISH) using a probe specific for ribosomal RNA of eubacteria (EUB338),<sup>48</sup> followed by immunofluorescence staining for  $\alpha$ -linked fucose residues by utilizing *Ulex europaeus* agglutinin I (UEA-I) lectin that allows the visualization of the mucus layer.<sup>50</sup> Whereas bacteria were clearly separated from the colonic epithelium in control mice, we found that bacteria penetrated into the compromised mucus layer and came in contact with the enterocyte surface in colons from *Xbp1*<sup>IEC-KO</sup> mice (Figure 4E). Taken together, these results showed that IEC-specific XBP1 ablation caused diminished MUC2 expression and compromised the formation of the protective mucus layer, allowing luminal bacteria to come in contact with the colonic epithelium.

Whereas bacteria were clearly separated from the colonic epithelium in control mice, we found that bacteria penetrated into the compromised mucus layer and came in contact with the enterocyte surface in colons from *Xbp1*<sup>IEC-KO</sup> mice (Figure 4E). Taken together, these results showed that IEC-specific XBP1 ablation caused diminished MUC2 expression and compromised the formation of the protective mucus layer, allowing luminal bacteria to come in contact with the colonic epithelium.

### *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> mice show translocation of bacteria into the colonic mucosa and systemic inflammation

We postulated that impaired mucus layer formation caused by XBP1 deficiency, combined with a breach in the epithelial barrier induced by necroptotic death of caspase-8-deficient IECs, could allow the penetration of bacteria into the mucosa and submucosa, resulting in severe colitis in *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> mice. First, we confirmed that *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> mice also displayed reduced MUC2 expression and diminished overall mucin levels (Figures 5A and S4A). In contrast to a previous report suggesting that IEC-specific caspase-8 ablation reduces MUC2 expression in the colon,<sup>43</sup> our RNA-seq and RT-qPCR analysis, as well as MUC2 immunostaining and Alcian blue-PAS staining, did not reveal a reduction in MUC2 expression or mucus levels in colons of *Casp8*<sup>IEC-KO</sup> compared with control mice (Figures 5A and S4A; Table S1). Thus, the reduced mucin levels in colons from *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> mice are caused by the loss of XBP1 but not caspase-8. To assess the translocation of bacteria into the subepithelial layers, we stained Swiss-roll colon sections from *Casp8*<sup>IEC-KO</sup> and *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> mice for bacterial 16S rRNA by FISH. Indeed, we detected the presence of bacteria within the submucosa in almost all *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> mice (10 out of 11 mice), as opposed to only 37.5% (9 out of 24) of *Casp8*<sup>IEC-KO</sup> mice (Figure 5B). Moreover, *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup>, but not *Casp8*<sup>IEC-KO</sup>, mice had increased spleen size and elevated numbers of granulocytes and monocytes in the peripheral blood (Figures 5C and 5D), indicating that bacterial translocation to the subepithelial layers triggered systemic immune activation. Collectively, these results demonstrated that combined IEC-specific ablation of XBP1 and caspase-8 caused translocation of bacteria from the lumen into the subepithelial colon layers and triggered systemic inflammation.

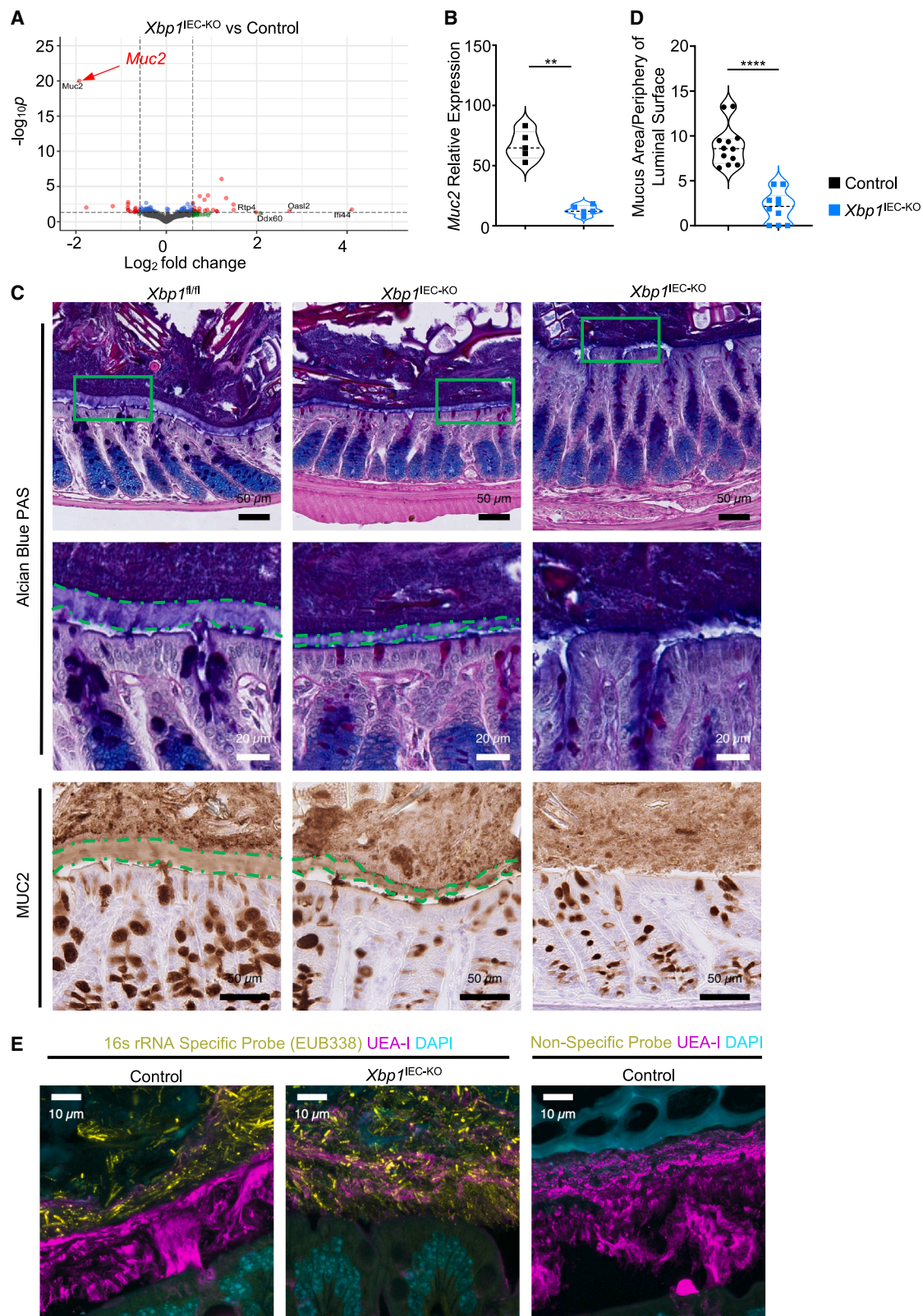
### Inhibition of necroptosis prevented bacterial translocation and systemic inflammation in *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> mice

MUC2 is exclusively produced and secreted by goblet cells in the colonic epithelium.<sup>8</sup> We wondered whether XBP1 deficiency

(B–D) Graphs depicting colon histopathology scores (B), the length of Ki67-positive crypt per entire crypt (C), and the percentage of mice bearing ulcers (D) of 8- to 12-week-old mice with the indicated genotypes. Violin plots show the median of biological replicates and the interquartile range of data. *p* values were calculated by non-parametric ANOVA. \**p* < 0.05. Each dot represents one mouse. The control, *Xbp1*<sup>IEC-KO</sup>, *Casp8*<sup>IEC-KO</sup>, and *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> groups originate from the same cohort of mice as those presented in Figure 1. The data are included here for direct comparison across different analyses.

(E) Heatmap depicting RNA-seq gene expression data from the distal colon of 8- to 12-week-old mice with the indicated genotypes (*n* = 5/genotype). The heatmap includes genes differentially regulated in *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> mice compared with wild-type controls (shrunk fold change of  $\geq 1.5$  or  $\leq 1.5$ , adjusted *p* value of  $\leq 0.05$ ). Columns represent individual mice, hierarchically clustered according to differential gene expression.

Sample sizes are indicated in Table S2.



**Figure 4. XBP1 expression in IECs regulates MUC2 expression and mucus layer formation in the colon**

(A) Volcano plot illustrating the RNA expression profile comparing the colons of 8- to 12-week-old *Xbp1*<sup>IEC-KO</sup> with control mice ( $n = 5/\text{genotype}$ ). Gray circles indicate genes are not significant; light green circles indicate genes with  $\log_2$  fold change  $\geq 1.5$  or  $\leq -1.5$ ; light blue circles indicate genes with  $p$  value of  $\leq 0.05$ ; light red circles indicate genes with  $\log_2$  fold change  $\geq 1.5$  or  $\leq -1.5$  and  $p$  value of  $\leq 0.05$ .

could induce goblet cell death, which could indirectly result in reduced MUC2 expression and a compromised mucus layer. We therefore assessed cell death in the colons of *Xbp1*<sup>IEC-KO</sup> and control mice by performing immunostaining for CC3 as well as TUNEL staining. We did not detect increased numbers of dying cells in the colons of *Xbp1*<sup>IEC-KO</sup> mice, arguing against the hypothesis that XBP1 deficiency might cause goblet cell death (Figure S5A). We then examined whether combined inhibition of caspase-8-induced apoptosis and RIPK3-MLKL-mediated necroptosis could rescue the impaired MUC2 expression and mucus layer formation caused by IEC-specific XBP1 deficiency. To this end, we performed MUC2 immunostaining and Alcian blue-PAS staining on colon sections from *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> *Ripk3*<sup>-/-</sup> and *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> *MLK1*<sup>-/-</sup> mice, as well as immunoblot analysis of MUC2 expression in IECs. These experiments showed that combined inhibition of apoptosis and necroptosis did not rescue the diminished MUC2 expression in *Xbp1*<sup>IEC-KO</sup> mice (Figures S5A–S5C). Furthermore, visualization and quantification of the mucus layer in colon cross-sections revealed that *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> *Ripk3*<sup>-/-</sup> and *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> *MLK1*<sup>-/-</sup> mice exhibited diminished mucus layer formation similar to that in *Xbp1*<sup>IEC-KO</sup> mice (Figures 6A and 6B). Taken together, these results showed that reduced mucin levels and impaired mucus layer formation in *Xbp1*<sup>IEC-KO</sup> mice were not caused by increased goblet cell necroptosis and/or apoptosis.

We then assessed whether necroptosis inhibition could prevent the infiltration of bacteria into the mucosa and submucosa of *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> *Ripk3*<sup>-/-</sup> and *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> *MLK1*<sup>-/-</sup> mice. In agreement with the reduced MUC2 expression, we found that bacteria penetrated into the impaired mucus layer and could reach the surface of the colonic epithelium of *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> *Ripk3*<sup>-/-</sup> and *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> *MLK1*<sup>-/-</sup> mice (Figure 6A). However, 16s rRNA FISH staining on colon Swiss-roll sections showed that bacteria did not infiltrate the colonic submucosa of *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> *Ripk3*<sup>-/-</sup> and *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> *MLK1*<sup>-/-</sup> mice (Figure 6C). Consistent with the absence of bacterial infiltration, *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> *Ripk3*<sup>-/-</sup> and *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> *MLK1*<sup>-/-</sup> mice did not display signs of systemic inflammation, as indicated by normal spleen size and similar numbers of granulocytes and monocytes in the peripheral blood compared with control littermates (Figure 6D). Taken together, these results showed that inhibition of necroptosis did not rescue the mucus layer defect but prevented the infiltration of bacteria in the subepithelial layers by restoring the epithelial barrier.

### XBP1 deletion reduces *Muc2* expression in a goblet-cell-intrinsic manner

The presence and composition of the intestinal microbiota regulate MUC2 production and the permeability and thickness of the mucus layer in the colon.<sup>50–53</sup> We therefore asked whether XBP1 deficiency caused reduced *Muc2* expression in a cell-intrinsic manner or indirectly by affecting other factors such as the microbiota. To address this question, we established colon organoids<sup>54</sup> from control as well as *Xbp1*<sup>IEC-KO</sup> mice and differentiated them into goblet cells by inhibiting Notch signaling with incubation with the  $\gamma$ -secretase inhibitor DAPT<sup>55,56</sup> (Figure 7A). RT-qPCR analysis revealed that DAPT treatment induced robust *Muc2* expression in colon organoids from control mice, which was considerably reduced in organoids from *Xbp1*<sup>IEC-KO</sup> mice (Figure 7B). XBP1 deficiency did not interfere with goblet cell differentiation, as indicated by similar expression of transcription factors important for secretory cell fate determination, including *Atoh1* (*Math1*), *Spdef*, and *Klf4*<sup>57–60</sup> in control and XBP1-deficient organoids (Figure 7B). These findings are consistent with unaltered goblet cell number in the colons of *Xbp1*<sup>IEC-KO</sup> mice (Figure S4C) and RNA-seq analysis showing that goblet cell differentiation genes were not reduced in the colons of *Xbp1*<sup>IEC-KO</sup> mice (Figure 4A; Table S1). Thus, XBP1 deficiency inhibited MUC2 expression in goblet cells in a cell-intrinsic manner.

IEC-specific XBP1 ablation led to the activation of inositol-requiring enzyme-1 $\alpha$  (IRE1 $\alpha$ ) as indicated by increased IRE1 $\alpha$  phosphorylation in colonic IECs from *Xbp1*<sup>IEC-KO</sup> compared with control mice (Figures S6A and S6B), in line with previous reports.<sup>44</sup> Autophosphorylation of IRE1 $\alpha$  induces its endoribonuclease-domain-mediated RNase activity, which mediates unconventional splicing of unspliced *Xbp1* (*Xbp1u*), leading to the generation of spliced *Xbp1* (*Xbp1s*). In addition to the unconventional splicing of *Xbp1u*, IRE1 $\alpha$  can degrade a certain pool of mRNAs via its endonuclease domain in a process called regulated IRE1-dependent decay (RIDD).<sup>61,62</sup> We therefore hypothesized that *Muc2* could be a target of RIDD by active IRE1 $\alpha$  in XBP1-deficient goblet cells. To test this hypothesis, we treated organoids from control or *Xbp1*<sup>IEC-KO</sup> mice with G'9668, a kinase inhibitor highly specific to IRE1 $\alpha$ <sup>63</sup> (Figure 7C). Cre-mediated excision of the loxP-flanked exon 2 of the *Xbp1* gene leads to a frameshift that introduces a premature stop codon, resulting in loss of XBP1S protein expression.<sup>41</sup> As the IRE1-mediated splicing site is retained upon exon 2 deletion and XBP1S ablation induces IRE1 activation, we could assess the activity of IRE1 by performing an *Xbp1* splicing assay in organoids from control or *Xbp1*<sup>IEC-KO</sup> mice<sup>41,64</sup> (Figure 7D). Detection of *Xbp1u* and

(B) Graphs depicting relative mRNA expression of *Muc2* measured by RT-qPCR in the colons of 8- to 12-week-old mice with the indicated genotypes. Violin plots show the median of biological replicates and the interquartile range of data.

(C) Representative images of Carnoy's-fixed colon sections from 11-week-old mice with the indicated genotypes stained with Alcian blue-PAS or immunostained for MUC2. The green dashed line indicates the mucus layer. Scale bars, 50  $\mu$ m (upper and lower) and 20  $\mu$ m (middle).

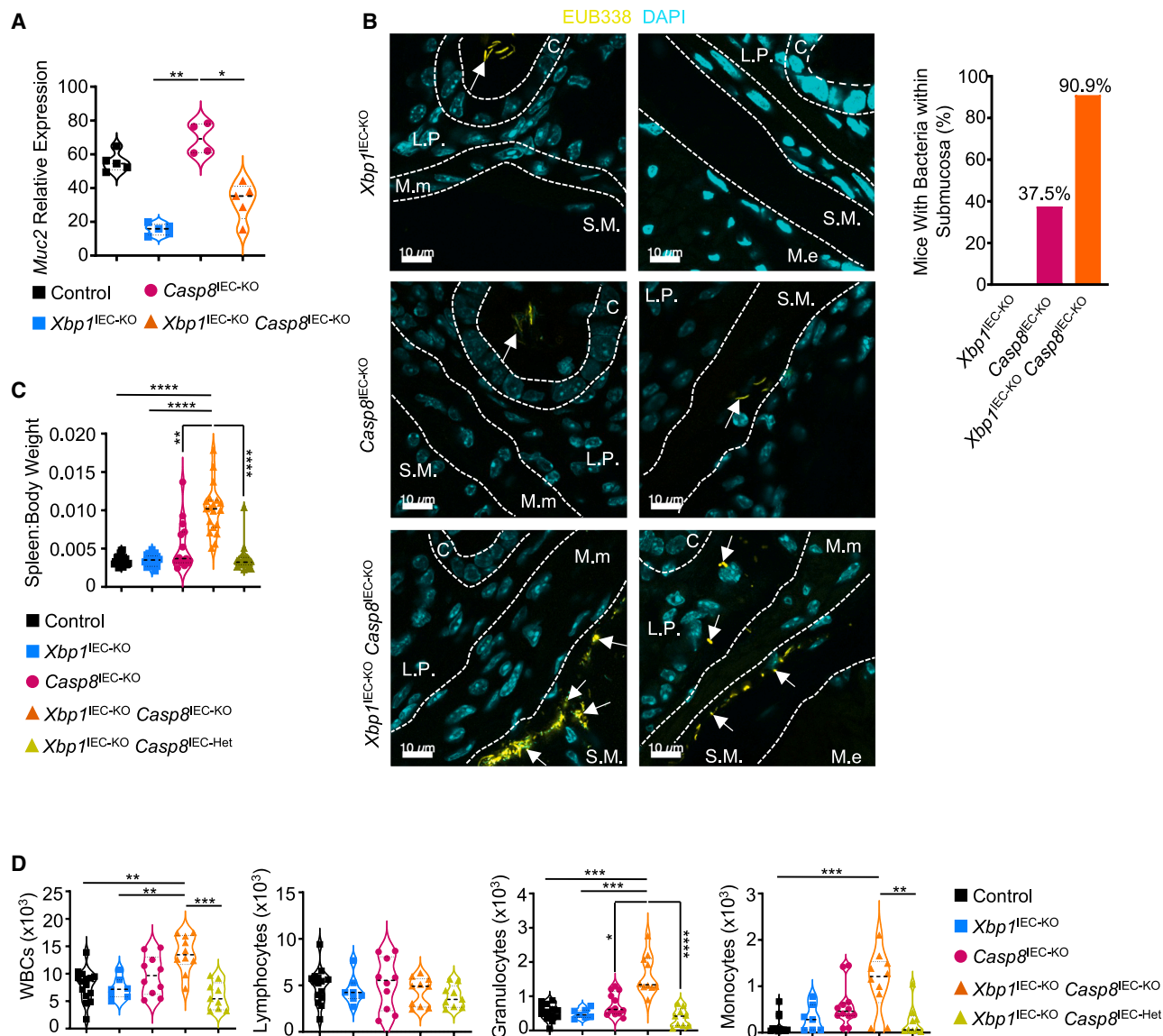
(D) Graphs depicting quantification of mucus layer thickness in colon sections from 11-week-old mice with the indicated genotypes. Violin plots show the median of biological replicates and the interquartile range of data.

(E) Representative images of Carnoy's-fixed colon tissue from 11-week-old mice with indicated genotypes stained with bacterial 16S rRNA *in situ* hybridization probe EUB338 (yellow) or non-specific control hybridization probe. Mucus was immunostained with UEA-I (purple), and nuclei were stained with DAPI (aqua). Scale bars, 10  $\mu$ m.

*p* values were calculated by non-parametric Mann-Whitney test. \*\**p* < 0.01, \*\*\*\**p* < 0.0001. Each dot represents one mouse.

Sample sizes are indicated in Table S2. Scale bars: 50  $\mu$ m (upper and lower), 20  $\mu$ m (middle) in (C), and 10  $\mu$ m in (E).



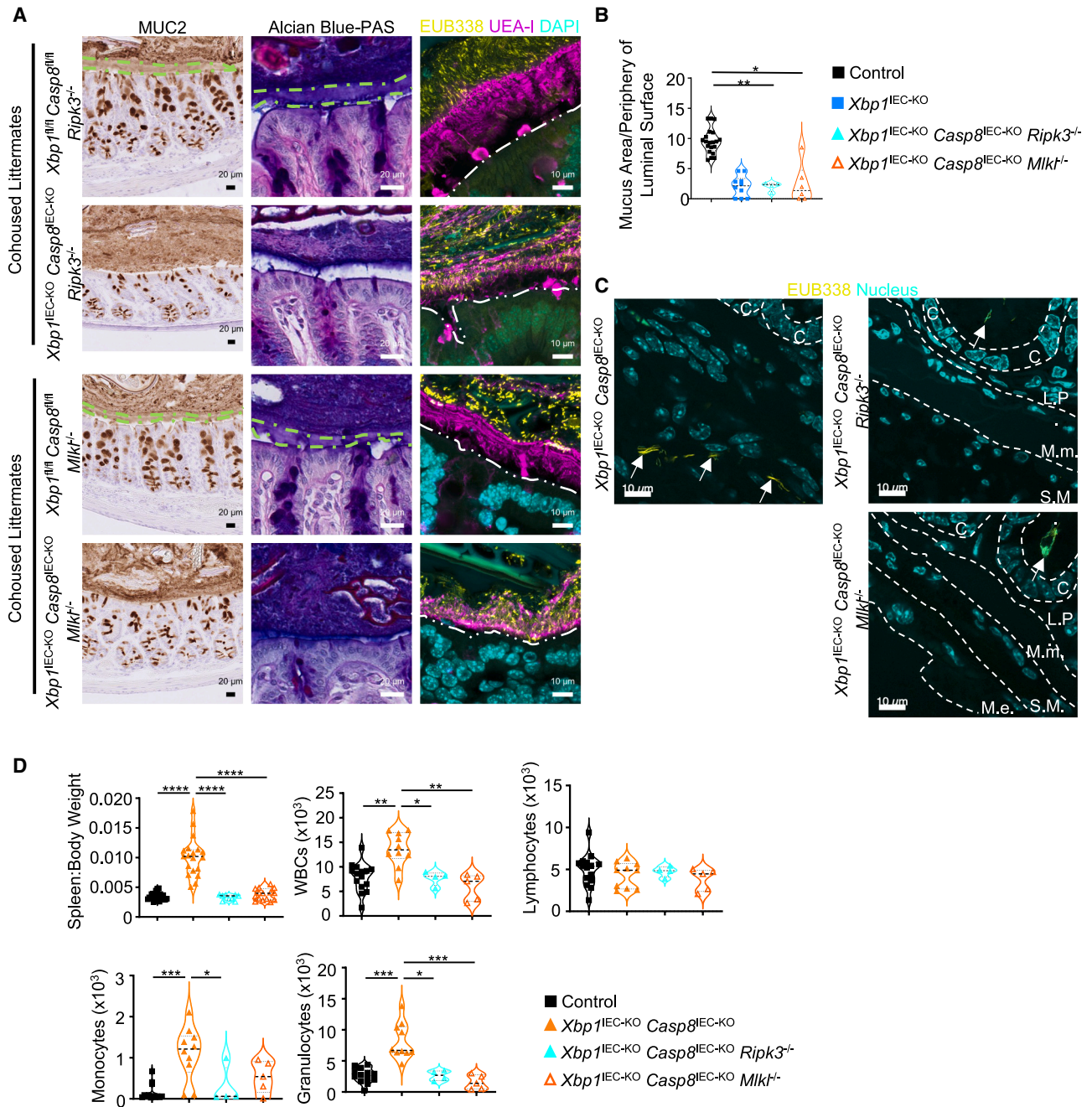


**Figure 5. Epithelial-specific XBP1 suppresses the translocation of bacteria and prevents systemic inflammation in *Casp8*<sup>IEC-KO</sup> mice**

(A) Graphs depicting relative mRNA expression of *Muc2* in the colons of 8- to 12-week-old mice with indicated genotypes. (B) Representative images of Swiss-roll colon sections from 8- to 12-week-old mice with the indicated genotypes stained with bacterial 16S rRNA *in situ* hybridization probe EUB338 (yellow). Nuclei were stained with DAPI (aqua). Each colonic structure and layer is labeled with white dashed lines. C, crypt; L.P, lamina propria; M.m, muscularis mucosae; S.M, submucosa; M.e, muscularis externa. White arrows mark bacteria. Bar graph depicting the percentage of 8- to 12-week-old mice with indicated genotypes that contain bacteria in the submucosa layer (right). Scale bars, 10  $\mu$ m. (C) Graphs showing the ratio of spleen weight to body weight of 8- to 12-week-old mice with the indicated genotypes. (D) Graphs depicting peripheral blood analysis of 8- to 12-week-old mice with the indicated genotypes, assayed by differential hematology analyzer. Violin plots show the median of biological replicates and the interquartile range of data. *p* values were calculated by non-parametric ANOVA. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.005, \*\*\*\**p* < 0.0001. Each dot represents one mouse. Sample sizes are indicated in Table S2. WBCs, white blood cells.

*Xbp1s* by RT-PCR revealed increased *Xbp1* splicing in XBP1-deficient organoids, which was suppressed by the IRE1 kinase inhibitor G'9668 (Figure 7D). However, RT-qPCR analysis revealed that *Muc2* mRNA expression in goblet-cell-differentiated organoids lacking XBP1 was not increased upon G'9668 treatment (Figure 7E). In addition to IRE1 $\alpha$ , mucosal epithelial cells express the IRE1 paralog IRE1 $\beta$  (gene name ERN2).<sup>65,66</sup> IRE1 $\beta$  expression is highly enriched in goblet cells<sup>67</sup> and plays an

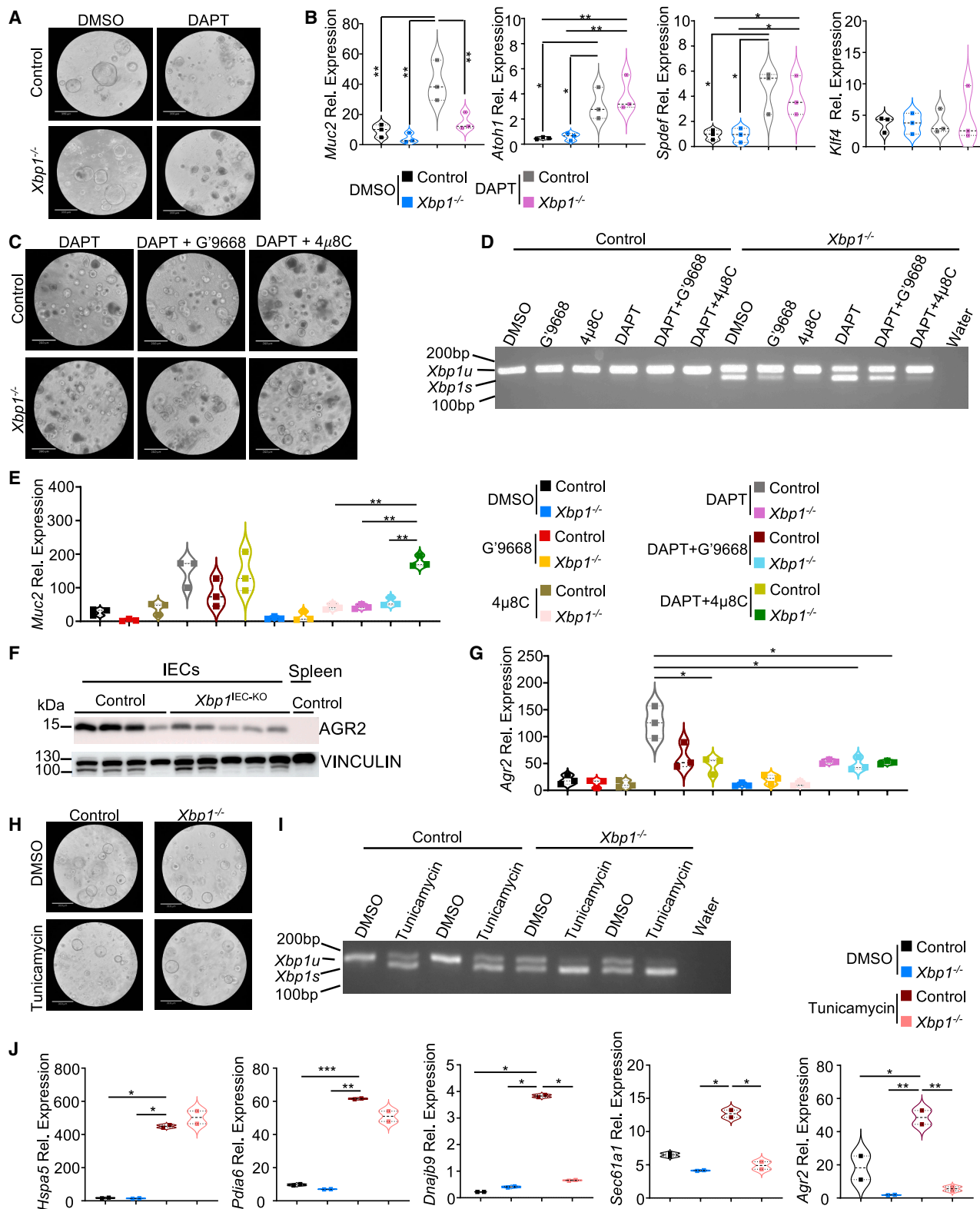
important role in the maintenance of mucosal homeostasis.<sup>68,69</sup> Similar to IRE1 $\alpha$ , IRE1 $\beta$  contains an endonuclease domain that can perform unconventional splicing of *Xbp1u*.<sup>70,71</sup> It is proposed that the endonuclease activity of IRE1 $\beta$  can degrade *Muc2* mRNA and contribute to intestinal mucin homeostasis.<sup>72</sup> In the *Xbp1* splicing assay, we detected a residual *Xbp1s* band in the presence of IRE1 $\alpha$  kinase inhibitor, G'9668 (Figure 7D). We therefore asked whether IRE1 $\beta$  could be activated and degrade *Muc2*



**Figure 6. Epithelial-specific XBP1 regulates *Muc2* expression independent of intrinsic apoptosis and necroptosis**

(A) Representative images of Carnoy's-fixed colon sections from 11-week-old mice with indicated genotypes immunostained for MUC2 (left) or stained with Alcian blue-PAS (middle) or bacterial 16S rRNA *in situ* hybridization probe EUB338 (yellow), UEA-I (purple), and DAPI (aqua). (Right) Mucus layers are indicated with green dashed lines. Intestinal epithelial borders in the right panel are indicated with white dashed lines. Scale bars: 20  $\mu$ m in left and middle, 10  $\mu$ m in right. (B) Graph depicting quantification of mucus layer thicknesses from 11-week-old mice with indicated genotypes. The control and *Xbp1<sup>IEC-KO</sup>* groups originate from the same cohort of mice as those presented in Figure 4D. The data are included here for direct comparison across different analyses. (C) Representative images of Swiss-roll colon sections from 8- to 12-week-old mice with the indicated genotypes that were stained with bacterial 16S rRNA *in situ* hybridization probe EUB338 (yellow). Nuclei were stained with DAPI (aqua). Each colonic structure and layer is labeled with white dashed lines. C, crypt; L.P, lamina propria; M.m, muscularis mucosae; S.M, submucosa; M.e, muscularis externa. White arrows mark bacteria. Scale bars, 10  $\mu$ m. (D) Graphs showing the ratio of spleen weight to body weight and peripheral blood analysis of 8- to 12-week-old mice with the indicated genotypes, assayed by differential hematology analyzer. The control and *Xbp1<sup>IEC-KO</sup> Casp8<sup>IEC-KO</sup>* groups originate from the same cohort of mice as those presented in Figure 5D. The data are included here for direct comparison across different analyses. WBCs, white blood cells.

Violin plots show the median of biological replicates and the interquartile range of data. *p* values were calculated by non-parametric ANOVA. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.005, \*\*\*\**p* < 0.0001. Each dot represents one mouse. Sample sizes are indicated in Table S2.



**Figure 7. XBP1 regulates *Muc2* expression in a goblet-cell-intrinsic manner**

(A) Representative images of mouse colon organoids treated with DMSO or DAPT for 6 days. Scale bars, 360  $\mu$ m.

(B) Graphs depicting relative mRNA expression of *Muc2*, *Atoh1*, *Klf4*, and *Spdef* in DMSO or DAPT-treated organoids for 6 days measured by RT-qPCR.

(C) Representative images of mouse colon organoids treated with G'9668 or 4 $\mu$ 8C for 24 h after being differentiated with DAPT for 5 days. Scale bars, 360  $\mu$ m.

(legend continued on next page)



mRNA upon XBP1 ablation in goblet cells. To address this question, we incubated goblet-cell-differentiated control or *Xbp1*<sup>-/-</sup> organoids with 4 $\mu$ 8C, an IRE1-endonuclease inhibitor, which was demonstrated to inhibit the endonuclease activities of both IRE1 $\alpha$  and IRE1 $\beta$ .<sup>73,74</sup> Indeed, 4 $\mu$ 8C treatment suppressed the activation of IRE1, as measured by the detection of *Xbp1* splicing in XBP1-deficient organoids (Figure 7D). Moreover, 4 $\mu$ 8C treatment restored the *Muc2* mRNA expression in *Xbp1*<sup>-/-</sup> organoids to control levels (Figure 7E). In addition, inhibition of IRE1 did not alter the DAPT-induced goblet cell differentiation in colon organoids (Figure S6C). Together, these results support that *Muc2* is degraded by IRE1 $\beta$  in goblet cells lacking XBP1.

Anterior-gradient protein homolog 2 (AGR2) is a member of the family of protein disulfide isomerases (PDIs)<sup>75</sup> and is linked with IBD.<sup>76</sup> *Agr2*<sup>-/-</sup> mice show epithelial ER stress, age-progressive rectal prolapse, and susceptibility to acute severe colitis upon DSS administration.<sup>77,78</sup> The expression of AGR2 is highly enriched in goblet cells, and the absence of AGR2 leads to reduced MUC2 production in goblet cells and impaired mucus layer formation in the colon, demonstrating that AGR2 plays a critical role in the maintenance of ER homeostasis and MUC2 biosynthesis in the intestinal epithelium.<sup>67,77–81</sup> Furthermore, XBP1 is proposed to regulate *Agr2* expression in lung epithelial cells.<sup>82</sup> AGR2 interacts with IRE1 $\beta$  and prevents its activation as well as IRE1 $\beta$ -mediated *Xbp1u* splicing.<sup>73,74</sup> We therefore reasoned that XBP1-deficient goblet cells might show impaired AGR2 expression, leading to activation of IRE1 $\beta$  and consequently *Muc2* mRNA decay. Indeed, immunoblot analysis revealed diminished AGR2 expression in IECs from *Xbp1*<sup>IEC-KO</sup> compared with control mice (Figure 7F). Consistent with this, goblet-cell-differentiated organoids lacking XBP1 had reduced *Agr2* mRNA expression compared with controls (Figure 7G). Although G'9668 and 4 $\mu$ 8C treatment did not alter *Agr2* expression in *Xbp1*<sup>-/-</sup> organoids, inhibiting IRE1 $\alpha$  kinase activity or the endonuclease activities of IRE1 $\alpha$  and IRE1 $\beta$  reduced *Agr2* expression in control organoids (Figure 7G), indicating that spliced XBP1 induces the expression of *Agr2*. IRE1 $\beta$  expression was not affected in XBP1-deficient IECs or *Xbp1*<sup>-/-</sup> organoids compared with their respective controls (Figures S6D and S6E). To study whether XBP1 regulates AGR2 expression, we chemically induced ER stress by incubating organoids with tunicamycin (Figure 7H). Increased *Xbp1u* splicing as well as elevated *Hspa5* (binding immunoglobulin protein [BiP]) mRNA expression confirmed the induction of ER stress in organoids treated with tunicamycin (Figures 7I and 7J). Furthermore, RT-qPCR analysis revealed that control and XBP1-deficient organoids showed strong induction of *Pdia6*, an activating tran-

scription factor 6 (ATF6)-mediated UPR target gene (Figure 7J). However, organoids lacking XBP1 failed to upregulate two known XBP1 target genes, *Dnajb9* and *Sec61a1* (Figure 7J). Moreover, *Agr2* expression was not increased by tunicamycin in *Xbp1*<sup>-/-</sup> organoids, in contrast to control organoids (Figure 7J). Collectively, our results suggest that XBP1 prevents activation of IRE1 $\beta$  and inhibits IRE1 $\beta$ -mediated *Muc2* mRNA decay by inducing AGR2 (Figure S6F).

## DISCUSSION

Several clinical and pre-clinical studies provided evidence supporting that disturbance of the epithelial barrier contributes to IBD pathogenesis.<sup>2,5,7</sup> Multiple pathways contribute to the formation and maintenance of a healthy epithelial barrier, but the crosstalk between the different pathways and the possible functional redundancies and synergistic effects have remained poorly understood. Here, we revealed a hitherto unacknowledged synergistic effect between ER stress and necroptosis regulation in the pathogenesis of chronic colon inflammation. Our experiments identified profound reduction of MUC2 expression and impaired formation of the colonic mucus barrier as the main mechanism by which XBP1 deficiency caused colitis exacerbation in *Casp8*<sup>IEC-KO</sup> and *Fadd*<sup>IEC-KO</sup> mice. Although *Casp8*<sup>IEC-KO</sup> and *Fadd*<sup>IEC-KO</sup> mice do develop colitis, the severity of the pathology varies between individual animals, with most mice showing mild inflammation at a young age. Additional XBP1 ablation in IECs led to a more severe colitis affecting most of the animals, suggesting that XBP1-mediated mucus barrier formation is critical to suppress colon inflammation when the epithelial monolayer is compromised due to ongoing necroptosis of colonocytes. As colitis development in *Fadd*<sup>IEC-KO</sup> mice depends on the microbiota,<sup>32</sup> our results suggest that the exacerbation of colitis in the *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> and *Xbp1*<sup>IEC-KO</sup> *Fadd*<sup>IEC-KO</sup> mice is caused by bacteria reaching the epithelial surface due to the mucus layer impairment. The role of bacteria is further supported by our findings that XBP1 deficiency did not aggravate the small intestinal phenotype of *Casp8*<sup>IEC-KO</sup> and *Fadd*<sup>IEC-KO</sup> mice, which does not depend on the microbiota.<sup>32</sup> Overall, our results suggest a model whereby formation of a healthy mucus layer that impedes bacterial contact with the epithelium has a critical function to suppress inflammation, even when the epithelial barrier is disrupted by death of IECs. Vice versa, the lack of spontaneous colitis in *Xbp1*<sup>IEC-KO</sup> mice shows that an intact epithelial monolayer is sufficient to prevent colitis development when the mucus layer is compromised. However, complete loss of the mucus layer in mice lacking

(D) Representative *Xbp1* splicing results in colon organoids treated with G'9668 or 4 $\mu$ 8C for 24 h after incubation with or without DAPT for 5 days.

(E) Graphs depicting relative mRNA expression of *Muc2* in colon organoids treated with G'9668 or 4 $\mu$ 8C for 24 h after incubation, with or without DAPT for 5 days, measured by RT-qPCR.

(F) Immunoblot analysis of colonic IECs or spleen protein lysates from 11-week-old mice of the indicated genotypes with the indicated antibodies. Lanes represent individual mice.

(G) Graphs depicting relative mRNA expression of *Agr2* in colon organoids treated with G'9668 or 4 $\mu$ 8C for 24 h after incubation, with or without DAPT for 5 days, measured by RT-qPCR.

(H–J) Representative images (H), *Xbp1* splicing results (I), and graphs depicting relative mRNA expression of *Hspa5*, *Pdia6*, *Dnajb9*, *Sec61a1*, and *Agr2* in colon organoids treated with DMSO or 10  $\mu$ g/mL tunicamycin for 5 h (J). Scale bars, 360  $\mu$ m.

Violin plots show the median of biological replicates and the interquartile range of data.

*p* values were calculated by one-way ANOVA t test with post hoc multiple test. \**p* < 0.05, \*\**p* < 0.01. Each dot in (B), (E), (G), and (J) represents an isolated organoid from one mouse for each genotype. Sample sizes are indicated in Table S2.

MUC2 causes spontaneous progressive colon inflammation,<sup>48,83,84</sup> highlighting an instance where a viable epithelial monolayer is no longer sufficient to prevent colitis when the entire mucus barrier is missing.

Our finding that XBP1 regulates the establishment of the colonic mucus layer by controlling the expression of MUC2 is consistent with earlier work showing that XBP1 as well as IRE1 $\beta$ /ERN2, a paralog of IRE1 $\alpha$  that is exclusively expressed in mucosal epithelial cells,<sup>65,66,69</sup> regulate the formation of the mucus barrier.<sup>68</sup> Our genetic studies provided evidence that the impaired mucus barrier in *Xbp1*<sup>IEC-KO</sup> mice is not induced by caspase-8-mediated apoptosis and RIPK3-MLKL-dependent necroptosis of goblet cells. Moreover, our results showing that XBP1 deficiency diminished *Muc2* expression in colonic organoids support that XBP1 regulates MUC2 production in goblet cells in a cell-intrinsic manner independently of the intestinal microbiota. In contrast to IRE1 $\beta$ -deficient organoids,<sup>68</sup> colon organoids lacking XBP1 showed normal differentiation into goblet cells, suggesting that IRE1 $\beta$  and XBP1 have different modes of action in goblet cells. Our results are consistent with a model whereby XBP1 regulates *Muc2* expression indirectly by inducing the expression of the IRE1 $\beta$  inhibitor AGR2. In the absence of XBP1, AGR2 expression is suppressed, resulting in increased IRE1 endonuclease activity, which leads to *Muc2* mRNA decay. These findings provide a functional link between XBP1 and AGR2 and contribute to the better understanding of the mechanisms by which these proteins are implicated in the pathogenesis of IBD.<sup>41,76</sup>

In contrast to the colon, XBP1 deficiency did not aggravate the development of ileitis in *Casp8*<sup>IEC-KO</sup> and *FADD*<sup>IEC-KO</sup> mice, suggesting that the XBP1 and FADD-caspase-8 pathways do not exert synergistic effects in the small intestine. Moreover, combined inhibition of caspase-8-mediated apoptosis and RIPK3-MLKL-mediated necroptosis could not restore the paneth cell defects of *Xbp1*<sup>IEC-KO</sup> mice. In addition, consistent with a recent report,<sup>85</sup> we did not detect increased numbers of TUNEL<sup>+</sup> or CC3<sup>+</sup> cells in *Xbp1*<sup>IEC-KO</sup> mice, arguing against cell death as a cause for the paneth cell impairment induced by XBP1 deficiency. However, a previous study reported that tamoxifen-inducible XBP1 deficiency in enterocytes causes increased numbers of TUNEL<sup>+</sup> cells in intestinal crypts,<sup>44</sup> suggesting that death of paneth cells might be more difficult to detect under constitutive XBP1 ablation as opposed to the inducible model.

Taken together, our results uncovered an important crosstalk between ER stress and necroptosis regulation in IECs for the maintenance of a healthy immune homeostasis and the prevention of inflammation in the intestine. These findings imply that redundancies between different pathways could underlie the low penetrance of most of the IBD susceptibility genes and suggest that combined defects affecting different components of the intestinal barrier may be required for the initiation and chronicity of intestinal inflammation.

### Limitations of the study

Our genetic studies revealed that caspase-8 and XBP1 cooperate to maintain mucosal immune homeostasis and prevent colon inflammation by controlling different components of the intestinal epithelial barrier. These results obtained in relevant mouse models suggest that a crosstalk between XBP1 and caspase-8 could be implicated in the pathogenesis of IBD. Although these

pathways are conserved between mice and humans, additional studies will be required to assess whether similar mechanisms regulate the epithelial barrier and mucosal immune homeostasis in humans. It will be important to determine whether MUC2 and AGR2 expression is reduced in the colonic epithelium of IBD patients with hypomorphic mutations in *XBP1*. Furthermore, studies in XBP1-deficient human colonic organoids will be valuable in assessing the role of XBP1 in controlling MUC2 and AGR2 expression in response to ER stress. Moreover, additional experiments such as chromatin immunoprecipitation sequencing (ChIP-seq) will be required to address whether XBP1 regulates AGR2 expression in goblet cells by direct transcriptional control or other mechanisms. Future studies addressing these questions will be critical to fully delineate the transcriptional network governed by XBP1 in regulating MUC2 expression, goblet cell physiology, and mucosal barrier integrity.

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources and reagents should be directed to, and will be fulfilled by, the lead contact, Manolis Pasparakis ([pasparakis@uni-koeln.de](mailto:pasparakis@uni-koeln.de)).

#### Materials availability

*Fadd*<sup>fl/fl</sup> and *Mkl1*<sup>-/-</sup> mice can be obtained from Manolis Pasparakis, Institute for Genetics, Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases (CECAD), University of Cologne, 50931 Cologne, Germany. *Casp8*<sup>fl/fl</sup> mice can be obtained from Stephen M. Hedrick, Division of Biological Sciences, University of California, San Diego, La Jolla, CA 92093, USA. *Tnfr1*<sup>fl/fl</sup> mice can be obtained from George Kollias, Biomedical Sciences Research Center "Alexander Fleming," Vari, GR-16672 Greece. *Tnfr1*<sup>fl/fl</sup> mice can be obtained from Sergei A. Nedospasov, Engelhardt Institute of Molecular Biology, Moscow, Russia. *Ripk3*<sup>-/-</sup> mice can be obtained from Vishva Dixit, Department of Physiological Chemistry, Genentech, South San Francisco, CA 94080. *Vil1-cre* mice can be obtained from Jackson Laboratories, JAX# 004586.

#### Data and code availability

The raw RNA-seq data have been deposited in the Gene Expression Omnibus (GEO) database under the accession number GEO: GSE244320. This paper does not report new original code.

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### AUTHOR CONTRIBUTIONS

G.G.K., R.S., M.D., K.V., and V.K. performed and analyzed experiments. U.G. analyzed RNA-seq data. S.A.N. contributed *Tnfr1*<sup>fl/fl</sup> mice. M.P. supervised the project. G.G.K. and M.P. interpreted the data and wrote the paper.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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## SUPPLEMENTAL INFORMATION

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE                                             | SOURCE                       | IDENTIFIER                        |
|-----------------------------------------------------------------|------------------------------|-----------------------------------|
| <b>Antibodies</b>                                               |                              |                                   |
| Anti-CD45                                                       | Thermo Fisher Scientific     | Cat# 14-0451-82; RRID: AB_467251  |
| Anti-cleaved-Caspase-3                                          | Cell Signaling Technology    | Cat# 9661; RRID: AB_2341188       |
| Anti-Lysozyme                                                   | Dako                         | Cat# F0372                        |
| Anti-Ki67                                                       | Abcam                        | Cat# ab15580; RRID: AB_443209     |
| Anti-MUC2                                                       | Abcam                        | Cat# ab272692; RRID: AB_2888616   |
| Anti-AGR2                                                       | Proteintech                  | Cat# 12275-1-AP; RRID: AB_2225096 |
| Anti-ERN2                                                       | Thermo Fisher Scientific     | Cat# PA5-116228; RRID: AB_2900862 |
| Anti-Phospho IRE1alpha-S724                                     | Sigma Aldrich                | Cat# SAB5700519; RRID: AB_3697855 |
| Anti-IRE1 (S724)                                                | Abcam                        | Cat# ab48187; RRID: AB_873899     |
| Anti-IRE1 $\alpha$                                              | Cell Signaling Technology    | Cat# 3293; RRID: AB_823545        |
| Anti-Vinculin                                                   | Cell Signaling Technology    | Cat# 9661; RRID: AB_2728768       |
| Anti-Rabbit-IgG (H+L), Biotin-XX                                | Thermo Fisher Scientific     | Cat# B-2770; RRID: AB_2536431     |
| Anti-Rat IgG (H+L), DSB-X Biotin                                | Thermo Fisher Scientific     | Cat# D-20697; RRID: AB_2536518    |
| ECL Anti-Rabbit IgG, HRP-linked                                 | Amersham                     | Cat# NA934; RRID: AB_772206       |
| <b>Chemicals, peptides, and recombinant proteins</b>            |                              |                                   |
| Ulex Europaeus Agglutinin I (UEA I), Biotinylated               | Vector Laboratories          | Cat# B-1065; RRID: AB_2336766     |
| Streptavidin, Alexa Fluor™ 488                                  | Thermo Fisher Scientific     | Cat# S11223                       |
| ReadyProbes™ In Situ Hybridization (ISH) Blocking Solution (5x) | Thermo Fisher Scientific     | Cat# R37620                       |
| 3dGRO® Wnt3a Conditioned Media Supplement                       | Sigma-Aldrich                | Cat# SCM112                       |
| Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix  | Corning                      | Cat# 354230                       |
| DMEM/F-12 with 15 mM HEPES                                      | StemCell                     | Cat# 36254                        |
| Y-27632 (Dihydrochloride)                                       | StemCell                     | Cat# 72304                        |
| DAPT                                                            | StemCell                     | Cat# 72082                        |
| 4 $\mu$ 8C                                                      | MedChem Express              | Cat# HY-19707                     |
| G'9668                                                          | Genentech Inc. <sup>63</sup> | N/A                               |
| Trypsin-EDTA (0.05%), phenol red                                | Thermo Fisher Scientific     | Cat# 25300054                     |
| SuperBlock™ T20 (TBS) Blocking-Buffer                           | Thermo Fisher Scientific     | Cat# 37536                        |
| ProLong™ Diamond Antifade Mountant with DAPI                    | Thermo Fisher Scientific     | Cat# P36962                       |
| Methanol, 99.9%, Extra Dry                                      | Thermo Fisher Scientific     | Cat# 326950010                    |
| Chloroform 99.0-99.4%                                           | AnalaR NORMAPUR ACS          | Cat# 22711290                     |
| Acetic acid, glacial 99.7+%                                     | Thermo Fisher Scientific     | Cat# 36289.AP                     |
| <b>Critical commercial assays</b>                               |                              |                                   |
| Streptavidin/Biotin Blocking Kit                                | Vector Laboratories          | Cat# SP-2002                      |
| ABC-HRP Kit, Peroxidase (Standard)                              | Vector Laboratories          | Cat# PK-6100                      |
| DAB Substrate Kit                                               | Abcam                        | Cat# ab64238                      |
| TrueVIEW® Autofluorescence Quenching Kit                        | Vector Laboratories          | Cat# SP-8500-15                   |
| Alcian Blue PAS Stain Kit                                       | Abcam                        | Cat# ab245876                     |
| TUNEL Assay Kit                                                 | Abcam                        | Cat# ab206386                     |
| IntestiCult™ Organoid Growth Medium                             | StemCell                     | Cat#06005                         |
| <i>Muc2</i> TaqMan gene expression assay                        | Thermo Fisher Scientific     | Assay ID: Mm01276696_m1           |
| <i>Defars10</i> TaqMan gene expression assay                    | Thermo Fisher Scientific     | Assay ID: Mm00833275_g1           |
| <i>Defa5</i> TaqMan gene expression assay                       | Thermo Fisher Scientific     | Assay ID: Mm00651548_g1           |
| <i>Lyz1</i> TaqMan gene expression assay                        | Thermo Fisher Scientific     | Assay ID: Mm00657323_m1           |
| <i>Atoh1</i> TaqMan gene expression assay                       | Thermo Fisher Scientific     | Assay ID: Mm00476035_s1           |

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**Continued**

| REAGENT or RESOURCE                                                                    | SOURCE                                | IDENTIFIER                    |
|----------------------------------------------------------------------------------------|---------------------------------------|-------------------------------|
| <i>Klf4</i> TaqMan gene expression assay                                               | Thermo Fisher Scientific              | Assay ID: Mm00516104_m1       |
| <i>Spdef</i> TaqMan gene expression assay                                              | Thermo Fisher Scientific              | Assay ID: Mm00600221_m1       |
| <i>Hspa5</i> TaqMan gene expression assay                                              | Thermo Fisher Scientific              | Assay ID: Mm00517691_m1       |
| <i>Tbp</i> TaqMan gene expression assay                                                | Thermo Fisher Scientific              | Assay ID: Mm00446973_m1       |
| <i>Muc2</i> IDT gene expression assay                                                  | IDT                                   | Assay ID: Mm.PT.58.29496069.g |
| <i>Agr2</i> IDT gene expression assay                                                  | IDT                                   | Assay ID: Mm.PT.58.43572495   |
| <i>Atoh1</i> IDT gene expression assay                                                 | IDT                                   | Assay ID: Mm.PT.58.29687973.g |
| <i>Gfi1</i> IDT gene expression assay                                                  | IDT                                   | Assay ID: Mm.PT.58.29190986   |
| <i>Spdef</i> IDT gene expression assay                                                 | IDT                                   | Assay ID: Mm.PT.58.31043304   |
| <i>Ern2</i> IDT gene expression assay                                                  | IDT                                   | Assay ID: Mm.PT.58.43462546   |
| <i>Pdia6</i> IDT gene expression assay                                                 | IDT                                   | Assay ID: Mm.PT.58.10390210   |
| <i>Dnajb9</i> IDT gene expression assay                                                | IDT                                   | Assay ID: Mm.PT.58.12562211   |
| <i>Sec61a1</i> IDT gene expression assay                                               | IDT                                   | Assay ID: Mm.PT.58.13207237   |
| <b>Deposited data</b>                                                                  |                                       |                               |
| 3' mRNA Sequencing                                                                     | This Paper                            | GEO: GSE244320                |
| <b>Experimental models: Organisms/strains</b>                                          |                                       |                               |
| Mouse: <i>Xbp1</i> <sup>fl/fl</sup> , <i>Xbp1</i> <sup>tm1a(EUCOMM)Wtsi</sup> C57BL/6N | EUCOMM                                | MGI:4432347                   |
| Mouse: <i>Villin1-Cre</i> : B6.Cg-Tg(Vil1-cre)997Gum/J, C57BL/6N                       | The Jackson Laboratory                | JAX# 004586                   |
| Mouse: <i>Casp8</i> <sup>fl/fl</sup> , <i>Casp8</i> <sup>tm1Hed</sup> , C57BL/6N       | Beisner et al. <sup>86</sup>          | MGI:3696985                   |
| Mouse: <i>Fadd</i> <sup>fl/fl</sup> , <i>Fadd</i> <sup>tm1Mpa</sup> , C57BL/6N         | Mc Guire et al. <sup>87</sup>         | MGI:3700154                   |
| Mouse: <i>Mik1</i> <sup>-/-</sup> , <i>Mik1</i> <sup>em1Mpa</sup> , C57BL/6N           | Lin et al. <sup>88</sup>              | MGI:6392916                   |
| Mouse: <i>Ripk3</i> <sup>-/-</sup> , <i>Ripk3</i> <sup>tm1Vmd</sup> , C57BL/6N         | Newton et al. <sup>89</sup>           | MGI:3028853                   |
| Mouse: <i>Tnfr1</i> <sup>fl/fl</sup> , <i>Tnfrsf1a</i> <sup>tm3Gkl</sup> , C57BL/6N    | Van Hauwermeiren et al. <sup>90</sup> | MGI:7563890                   |
| Mouse: <i>Tnf</i> <sup>fl/fl</sup> , <i>Tnf</i> <sup>tm1.1Sned</sup> , C57BL/6N        | Grivennikov et al. <sup>91</sup>      | MGI:3527143                   |
| <b>Oligonucleotides</b>                                                                |                                       |                               |
| EUB338:5TexRd-XN/GCTGCCTCCCGTAGGAGT                                                    | IDT                                   | N/A                           |
| Non-EUB338: 5TexRd-XN/ACTCCTACGGGAGGCAGC                                               | IDT                                   | N/A                           |
| <i>Xbp1</i> splicing assay Forward Primer:<br>5'-ACACGCTTGGGAATGGACAC-3'               | IDT                                   | N/A                           |
| <i>Xbp1</i> splicing assay Reverse Primer:<br>5'-CCATGGGAAGATGTTCTGGG-3'               | IDT                                   | N/A                           |
| <b>Software and algorithms</b>                                                         |                                       |                               |
| GraphPad Prism                                                                         | Graphpad Inc                          | RRID: SCR_002798              |
| QuPath                                                                                 | Bankhead et al. <sup>92</sup>         | RRID: SCR_018257              |
| Subread                                                                                | Liao et al. <sup>93</sup>             | RRID: SCR_009803              |
| Bioconductor                                                                           | Huber et al. <sup>94</sup>            | RRID: SCR_006442              |

**EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS**

**Mice**

*Casp8*<sup>fl/fl</sup>,<sup>86</sup> *Fadd*<sup>fl/fl</sup>,<sup>87</sup> *Villin-Cre*,<sup>95</sup> *Tnfr1*<sup>fl/fl</sup>,<sup>90</sup> *Tnf*<sup>fl/fl</sup>,<sup>91</sup> *Mik1*<sup>-/-</sup>,<sup>88</sup> and *Ripk3*<sup>-/-</sup>,<sup>89</sup> mice were described previously. *Xbp1*<sup>fl/fl</sup> mice were generated by crossing *Xbp1*<sup>tm1a(EUCOMM)Wtsi</sup> mice with *FLPe-Deleter* mice<sup>96</sup> to delete the FRT-flanked cassette. Mice used in this study were maintained in the animal facility of the CECAD Research Center, University of Cologne, in individually ventilated cages (Tecniplast, Greenline GM500) at 22°C (±2°C) and a relative humidity of 55% (±5%) under a 12-h light/12-h dark cycle on sterilized bedding (Aspen wood, Abedd) with access to a sterilized commercial pelleted diet (Ssniff Spezialdiäten) and acidified water *ad libitum*. The microbiological status of the mice was examined as recommended by the Federation of European Laboratory Animal Science Associations (FELASA), and the mice were free of all listed pathogens. All animal procedures were conducted in accordance with European, national and institutional guidelines, and protocols were approved by local government authorities (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen). Animals requiring medical attention were provided with appropriate care and were killed when they reached predetermined criteria of disease severity. No other exclusion criteria existed. Experimental

groups were not randomized as mice were assigned to groups based on genotype. The sample size was estimated based on previous experience. Female and male mice of the indicated genotypes were assigned to groups at random. Mice were analysed at the age stated in the respective figure legends. Mouse studies as well as immunohistochemical assessments of pathology were performed in a blinded fashion.

### Tissue Preparation

After the dissection of the sacrificed mice, the colon and small intestine were carefully removed from the rest of the body and placed into ice-cold PBS. ~0.5–1 cm pieces of the distal colon and small intestine, located just above the caecum, were snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until RNA expression analysis was performed. The colon and small intestines were longitudinally opened and were washed with ice-cold PBS. The remaining colon tissue and ~10–15 cm of the distal small intestinal tissues were Swiss-rolled and fixed in 4% PFA at  $4^{\circ}\text{C}$  overnight. For colonic mucus studies, the distal colons containing faecal material were excised from the rest of the colon and fixed in Carnoy's fixative (60% (v/v) Extra Dry Methanol, 30% (v/v) Chloroform, 10% (v/v) Glacial Acetic Acid) at  $4^{\circ}\text{C}$  for 7 days.

### Blood Analysis

Peripheral blood from the left ventricle was collected into EDTA-containing tubes. Blood-cell-counting analyses were performed by Abacus Junior Vet Analyser utilizing 40  $\mu\text{l}$  of collected blood, according to the manufacturer's instructions.

### Histology and Immunohistochemistry

After samples from mice were fixed in 4% PFA, they were embedded in paraffin. Sections of 3  $\mu\text{m}$  were subjected to histological analysis by haematoxylin and eosin (H&E) staining. To detect DNA fragmentation, TUNEL assay was performed by using a commercial kit (Abcam, ab206386) according to the manufacturer's manual. For immunohistochemical analysis, slides were rehydrated and incubated with peroxidase blocking buffer (0.04 M Sodium Citrate, 0.121 M  $\text{Na}_2\text{HPO}_4$ , 0.03 M  $\text{NaN}_3$ , 3%  $\text{H}_2\text{O}_2$ ) for 10 minutes. Slides were washed and antigen retrieval was performed by digestion with proteinase K (10  $\mu\text{g}/\text{ml}$ ) for 5 minutes in TEX-buffer (50 mM Tris, 1 mM EDTA, 0.5% Triton X-100, pH 8.0) for CD45 and lysozyme or with Sodium citrate buffer in a pressure cooker for 20 minutes for cleaved caspase-3, Ki67 or with Tris-EDTA buffer in a pressure cooker for 20 minutes for MUCIN2. Sections were washed with TBS supplemented with 0.1% Tween-20 (v/v) (TBS-T) and blocked for 60 minutes using commercial blocking buffer (ThermoFisher, cat no. 37536) supplemented with biotin from (1:100 dilution) from Avidin/Biotin blocking kit (Vector, cat no. SP-2001) to block endogenous avidin. After sections were briefly washed with TBS-T, they were incubated with the primary antibody CD45 (eBioscience, cat no. 14-0451), cleaved caspase-3 (CST, cat no. 9661), lysozyme (Dako, cat no. F0372) and avidin (1:100 dilution) from Avidin/Biotin blocking kit (Vector, cat no. SP-2001) overnight at  $4^{\circ}\text{C}$ . A biotinylated secondary anti-rat antibody (Invitrogen, cat no. D20697) or anti-rabbit (Invitrogen, cat no. B2770) was incubated on the sections for 60 minutes. Stainings were visualized using the ABC Vectastain Elite Kit (Vector Laboratories) and DAB substrate (Abcam, cat no. ab64238). Incubation times with DAB substrate were equal for all samples within one staining. Sections were then counterstained with haematoxylin for staining of the nuclei, dehydrated and mounted with Entellan. Histological sections were scanned using a Nanozoomer S360 (Hamamatsu) slide scanner. Images were analysed and processed using the Omero software package (<https://openmicroscopy.org>) and NDP.view2 Viewing software (Hamamatsu).

## METHOD DETAILS

### Mucus Layer Staining

After tissues were fixed in Carnoy's fixative, sections were washed two times with dry methanol for 30 minutes each, followed by two times with absolute ethanol for 20 minutes each. After the ethanol step, the tissues were cleared by incubating two times in xylene for 15 minutes each. The cleared tissues were incubated in melted paraffin for 15 minutes and embedded in paraffin. Sections of 5  $\mu\text{m}$  were stained with Alcian Blue and PAS (Abcam, cat no. ab245876). In short, slides were rehydrated and incubated with 3% acetic acid for 2 minutes. Excess acetic acid was removed, and slides were incubated with Alcian Blue for 8 minutes. After the slides were washed 2 times with distilled water, Periodic Acid was applied to them for 5 minutes. Periodic Acid was washed with distilled water. Slides were incubated with Schiff's solution for 5 minutes. Slides were then washed under running tap water, followed by 2 changes of distilled water. Sections were then counterstained with hematoxylin for staining of the nuclei, dehydrated and mounted with Entellan.

### FISH and Immunofluorescence

Sections fixed in Carnoy's Fixative were deparaffinized by pre-warmed xylene ( $60^{\circ}\text{C}$ ) for 10 minutes. After incubation with absolute ethanol for 5 minutes, slides were incubated with either 0.5  $\mu\text{g}$  EUB338 or non-specific probes, suspended in FISH hybridization solution (20 mM Tris-HCl, pH 7.4; 0.9 M NaCl; 0.1% (w/v); Sodium Dodecylsulfate (SDS) in nuclease-free water), at  $50^{\circ}\text{C}$  for 3 hours. Then slides were washed with FISH washing buffer (20 mM Tris-HCl, pH 7.4; 0.9 M NaCl; 0.1% (w/v)) at  $50^{\circ}\text{C}$  for 5 minutes. After the washing step, sections were incubated with biotinylated *Ulex europaeus* agglutinin I (UEA-I) (Vector Labs, B-1065-2) at RT for 1 hour. Sections were washed with TBS and incubated with Streptavidin, Alexa Fluor™ 488 conjugate (Invitrogen, S11223) at RT for 1 hour. Excess Streptavidin was washed, and sections were mounted with ProLong™ Diamond Antifade Mountant (Invitrogen, P36962).



Bacteria within the submucosa were detected by using 4% PFA-fixed Swiss rolled sections. Slides were rehydrated and incubated with either 0.5  $\mu$ g EUB338 or non-specific probes, suspended in FISH hybridization solution at 50°C for 3 hours. Then sections were washed with FISH washing buffer at 50°C for 5 minutes. After washing, sections were quenched using a commercial kit, TrueVIEW® Autofluorescence Quenching Kit (Vector Labs, SP-8400-15). Slides were washed with TBS and incubated with Höchst 3342 (Invitrogen, H3570) in TBS at RT for 20 minutes. Excess Höchst 3342 was washed, and sections were mounted with ProLong™ Diamond Antifade Mountant (Invitrogen, P36962). Sections were analysed and representative images were taken using LSM980 Airyscan 2 (Carl Zeiss).

### Histopathological analysis

The histopathological score was evaluated using 3  $\mu$ m thick H&E-stained sections of paraffin-embedded Swiss-rolled intestinal tissues, using a modified version of the scoring system described in.<sup>44</sup> Briefly, histopathology scores consist of four parameters: epithelial hyperplasia, quantity, and localization of tissue inflammation, epithelial cell death, and epithelial injury. An “area factor” for a fraction of affected tissue was considered and multiplied with the respective parameter score. Area factor is 1, 2, 3, and 4 for 0–25%, 25–50%, 50–75%, and 75–100% of the affected tissue in terms of the aforementioned parameters, respectively. The histology score is the sum of all their area factors with corresponding parameter scores, and the highest hypothetical score can be up to 64. Histological scores were based on one Swiss roll section per mouse and were performed in a blinded fashion.

MUC2-positive goblet cell counting, MUC2-positive theca area measurement, and crypt length analysis were performed using the QuPath program on Swiss-rolled colon sections immunostained for MUC2. Quantifications were conducted on at least 15 well-defined crypts in the distal colon of each mouse.

### Isolation of colonic crypts and colon organoid culture

Colon tissues from sacrificed mice were harvested, opened longitudinally, and luminal contents were cleaned with ice-cold DPBS for 2 times. The colon samples were cut into 2–5mm pieces and collected in ice-cold DPBS in 50 ml tubes. The colon pieces were washed with ice-cold DPBS by gently pipetting with serological pipette. Pieces were allowed to settle down and supernatants were removed. The pieces were washed for 15 times. Pieces were transferred into 50 ml tube containing colonic crypt isolation buffer ( $\text{Na}_2\text{HPO}_4$  [5.6 mM],  $\text{KH}_2\text{PO}_4$  [8mM], NaCl [96 mM], KCl [1.6 mM], Sucrose [44 mM], D-sorbitol [54.8 mM], DL-dithiothreitol [0.5 mM], EDTA [5 mM]) and incubated 4°C on rocker for 1 hour. After the incubation, pieces were allowed to settle down, and supernatants were removed. Pieces were suspended in 10 ml ice-cold DPBS containing 10% FCS. Intestinal pieces were pipetted up and down for 3 times and allowed to settle down. Supernatants were collected and passed through 70  $\mu$ m filter into 50 ml tubes. The colon pieces were resuspended in 10 ml ice-cold DPBS containing 10% FCS, and the collection of crypt fractions was repeated for 3 times. Supernatants containing crypt fractions were centrifuged at 150  $\times g$  for 5 min at 4°C. Crypts were resuspended in 10 ml DMEM/F-12 with 15 mM HEPES and centrifuged at 100  $\times g$  for 5 min at 4°C. Crypts were suspended in room temperature IntestiCult™ Organoid Growth Medium (StemCell, 06005) supplemented with Wnt3a conditioned medium (Sigma-Aldrich, SCM112). Crypts were mixed with ice-cold Matrigel (Corning) at a 1:1 ratio and plated in 48-well plates in 20  $\mu$ l drops. The plates were inverted to make domes and incubated in a CO<sub>2</sub> incubator (5% CO<sub>2</sub> at 37°C) for 30 minutes. After incubation, 250  $\mu$ l of IntestiCult™ Organoid Growth Medium supplemented with Wnt3a-conditioned medium was added to each well. Anoikis was prevented for 1–2 days of culture by adding 10  $\mu$ M Y-27632 (Dihydrochloride) (StemCell, Cat# 72304). Culture media were exchanged every 2–3 days. After 7–9 days, organoids were passaged by utilizing Trypsin-EDTA (0.05%), phenol red (Thermo Fisher Scientific, 25300054) containing 10  $\mu$ M Y-27632 (Dihydrochloride) to dissociate into single crypt fragments.

### Colon organoid experiments and RNA isolation

After passaging, control and *Xbp1*<sup>-/-</sup> organoids were plated in 48-well plates (20  $\mu$ l/well) and cultured with IntestiCult™ Organoid Growth Medium supplemented with Wnt3a conditioned medium containing 10  $\mu$ M Y-27632 (Dihydrochloride). The next day, organoids were treated with either DMSO (1:1000 dilution) or 10  $\mu$ M DAPT (StemCell, 72082). Every 2 days, corresponding media were refreshed. After 6 days, the media were removed, and Matrigel-Organoids were directly lysed with RNAzol® RT (Sigma-Aldrich, R4533-200ML). For IRE1 inhibitor experiments, organoids were treated with either 10  $\mu$ M G'9668 or 10  $\mu$ M 4 $\mu$ 8C on the 5th day. After 24 hours, Matrigel-Organoids were directly lysed with RNAzol® RT. For tunicamycin-induced ER stress experiments, undifferentiated, 24h post-passaged colon organoids were used. Organoids were treated with 10  $\mu$ g/ml Tunicamycin for 5 hours, and Matrigel-Organoids were directly lysed with RNAzol® RT. Proteins were removed by adding Molecular Grade water (200  $\mu$ l per 1 ml of RNAzol® RT) followed by centrifugation at 12000  $\times g$  for 15 minutes. Supernatants containing RNA were collected, and total RNAs were extracted with the Direct-zol RNA Miniprep Kit (Zymo Research, R2052) according to the manufacturer's instructions.

### Gene expression analysis by qRT-PCR

Total RNA was extracted with the Direct-zol RNA Miniprep Kit (Zymo Research, R2052) by using RNAzol® RT (Sigma-Aldrich, R4533), according to the manufacturer's instructions, followed by cDNA synthesis using the LunaScript RT SuperMix kit (New England Biolabs, E3010L). qRT-PCR was performed using TaqMan or IDT probes and Luna Universal Probe qPCR Master Mix (New England Biolabs, M3004X) in a QuantStudio 5 Real-Time PCR System (ABI). Reactions were run with *Tbp* as a reference gene. Relative expression of transcripts is shown using the 2<sup>- $\Delta\Delta\text{Ct}$</sup>  method and is represented in Box-Violin plots as mRNA expression values relative to the reference gene. The TaqMan probes used were as follows: *Muc2* (Mm01276696\_m1), *Defars10* (Mm00833275\_g1),

*Defa5* (Mm00651548\_g1), *Lyz1* (Mm00657323\_m1), *Tff3* (Mm00495590\_m1), *Atoh1* (Mm00476035\_s1), *Klf4* (Mm00516104\_m1), *Spdef* (Mm00600221\_m1), *Ccl2* (Mm00441242\_m1), *Cxcl1* (Mm00445235\_m1), *Il1b* (Mm00434228\_m1), *Hspa5* (Mm00517691\_m1), *Tbp* (Mm00446973\_m1). The IDT probes were: *Muc2* (Mm.PT.58.29496069.g), *Agr2* (Mm.PT.58.43572495), *Atoh1* (Mm.PT.58.29687973.g), *Gfi1* (Mm.PT.58.29190986), *Spdef* (Mm.PT.58.31043304), *Ern2* (Mm.PT.58.43462546), *Pdia6* (Mm.PT.58.10390210), *Dnajb9* (Mm.PT.58.12562211), *Sec61a1* (Mm.PT.58.13207237).

### Xbp1 splicing assay

cDNA synthesis was performed from 1 µg of total RNA extracted from stimulated colon organoids. Xbp1s and Xbp1u were amplified by using primers 5'-ACACGCTTGGGAATGGACAC-3' and 5'-CCATGGGAAGATGTTCTGGG-3' as described previously.<sup>41</sup> Products were resolved on 2% Agarose gel.

### IEC isolation and Immunoblot analysis

IECs were isolated by subsequent incubation of intestinal tissue in 1 mM dithiothreitol (DTT) and 1.5 mM EDTA solutions. Then, IECs were lysed in 20 mM Tris-HCl pH 7.5, 135 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 1% Triton X-100, 10% glycerol, phosphatase inhibitor (Roche), and cOmplete Mini Protease Inhibitor Cocktail (Roche) for 30 minutes on ice. Proteins were extracted by centrifugation at 18,000 × g at 4°C for 20 min. Protein concentrations were assessed, and 20–30 µg of proteins were denatured in LDS sample buffer (Thermo Fisher Scientific, NP0007) supplemented with β-mercaptoethanol at 70°C for 10 minutes. Cell lysates were separated on SDS-PAGE and transferred to PVDF membranes (IPVH00010, Millipore). Primary antibodies used for immunoblot analysis: AGR2 (Proteintech, 12275-1-AP), ERN2/IRE1beta (Thermo Fisher Scientific, PA5-116228), Phospho IRE1alpha-S724 (Sigma Aldrich, SAB5700519), IRE1α (Cell Signaling Technology, 3293), Vinculin (Cell Signaling Technology, 9661), ECL Anti-Rabbit IgG, HRP-linked (Amersham, NA934). For MUC2 immunoblot analysis, lysed IECs and whole spleen lysates were separated on NuPAGE™ 3 to 8%, Tris-Acetate, 1.0 mm, Mini Protein Gels (Thermo Fisher Scientific, WG1602), and MUC2 primary antibody was used (Abcam, ab272692).

### RNA sequencing analysis

Total RNA was extracted with the Direct-zol RNA Miniprep Kit by using RNAzol® RT. For each mouse, 2 µg total RNA (OD260/280 = 1.8–2.1, OD260/230 > 1.5 and RIN > 7) was used. The library preparation was performed using the QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen).

Single-end RNA-seq reads were produced by the Lexogen QuantSeq FWD protocol and sequenced on a NovaSeq 6000 instrument. Reads of the same sample sequenced on different lanes of the instrument were merged without correction for a batch effect. The Illumina TruSeq adapter and poly-A runs of length ≥ 18 were removed from the sequenced reads using cutadapt version 3.2.

Reads were aligned against *Mus musculus* GRCm38 (Ensembl v100) plus the ERCC92 spike-in sequences. The subread suite of programs<sup>93</sup> version 2.0.1 was used to produce a table of read counts per gene, with Ensembl *Mus musculus*.GRCm38.100.gtf as the genome annotation. Parameters for subread-align were -t 0 -multiMapping -B 5 -sortReadsByCoordinates. Parameters for featureCounts were -F "GTF" -t "exon" -g "gene\_id" -minOverlap 20 -M -primary -O -fraction -J -Q 30.

Differential gene expression and Gene Ontology over-representation analyses were performed within the R programming environment version 4.0.0, using packages of the Bioconductor system<sup>94</sup> version 3.11.

The initial set of 55579 genes was reduced to 15458 genes by the filter function of the Bioconductor package edgeR<sup>97</sup> version 3.30.3, with default parameters. DESeq2<sup>98</sup> version 1.28.1 was subsequently run on the filtered count table. The raw log<sub>2</sub> fold changes from this analysis were “shrunk” using the apeglm method<sup>99</sup> provided with the DESeq2 package. (Shrinkage pushes the log<sub>2</sub> fold changes of highly variable genes towards zero.) Normalized read counts were generated by DESeq2’s “counts” function with the parameter “normalized=TRUE”. This function divides the raw counts by sample-specific size factors, thereby correcting for sample-specific library sizes. Variance stabilized counts on the log scale for use in heatmaps were generated by the “rlog” function of DESeq2 with parameter blind=FALSE.

For Gene Ontology over-representation analysis (ORA), genes with a DESeq2 adjusted *p*-value < 0.05 and an absolute value of the shrunk log<sub>2</sub> fold change > log<sub>2</sub>(1.5) were selected and divided into an up-regulated and a down-regulated set. Both sets were separately used to query the Biological Process, Molecular Function, and Cellular Component sections of the Gene Ontology database, as provided by the Bioconductor annotation package org.Mm.eg.db (10.18129/B9.bioc.org.Mm.eg.db) version 3.12.0. The enrichGO function of the clusterProfiler<sup>100</sup> version 3.16.1, which is provided with Bioconductor, was used for the queries.

All visualizations (heatmaps, volcano plots and Gene Ontology concept networks (“emapplots”)) were prepared in R-4.1.0, using Bioconductor version 3.14, for reasons of better graphics quality.

Heatmaps of z-score-normalized rlog values were drawn using the Bioconductor package ComplexHeatmap<sup>101</sup> version 2.10.0. All heatmaps were restricted to genes with a DESeq2 adjusted *p*-value < 0.05 and an absolute value of the shrunk log<sub>2</sub> fold change > log<sub>2</sub>(1.5) in the contrast *Xbp1*<sup>IEC-KO</sup> *Casp8*<sup>IEC-KO</sup> versus control. Note that rlog values were computed on the full matrix, before extracting the sub-matrix containing the genes of interest.

ORA results were visualized as concept networks (“emapplots”), using the enrichplot package version 1.14.1, which is part of clusterProfiler.

## QUANTIFICATION AND STATISTICAL ANALYSIS

Areas of the mucus layers and the periphery of the epithelium were calculated by the QuPath program. All statistical analysis was performed with GraphPad Prism. Body weights over age were compared using the two-way ANOVA. A non-parametric Mann–Whitney test, Ordinary one-way ANOVA with Tukey’s multiple-comparisons test or Kruskal–Wallis test corrected by Dunn’s multiple-comparisons test were performed. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.005$ , \*\*\*\* $p < 0.0001$  for all figures. D’Agostino–Pearson omnibus normality test was performed for each graph to test whether the data follow Gaussian Distribution. Violin plots show the median of biological replicates and the interquartile range of data. No statistical method was used to predetermine sample size. No mice samples were excluded.