

# Defective ATG16L1-mediated removal of IRE1 $\alpha$ drives Crohn's disease-like ileitis

Markus Tschurtschenthaler,<sup>1</sup> Timon E. Adolph,<sup>1\*</sup> Jonathan W. Ashcroft,<sup>1\*</sup> Lukas Niederreiter,<sup>1\*</sup> Richa Bharti,<sup>3\*</sup> Svetlana Saveljeva,<sup>1</sup> Joya Bhattacharyya,<sup>1</sup> Magdalena B. Flak,<sup>4</sup> David Q. Shih,<sup>5</sup> Gwenny M. Fuhler,<sup>6</sup> Miles Parkes,<sup>1</sup> Kenji Kohno,<sup>7</sup> Takao Iwawaki,<sup>8</sup> C. Janneke van der Woude,<sup>6</sup> Heather P. Harding,<sup>2</sup> Andrew M. Smith,<sup>9</sup> Maikel P. Peppelenbosch,<sup>6</sup> Stephan R. Targan,<sup>5</sup> David Ron,<sup>2</sup> Philip Rosenstiel,<sup>3</sup> Richard S. Blumberg,<sup>4\*\*</sup> and Arthur Kaser<sup>1\*\*</sup>

<sup>1</sup>Division of Gastroenterology and Hepatology, Department of Medicine and <sup>2</sup>Cambridge Institute for Medical Research, University of Cambridge, Cambridge CB2 0QQ, England, UK

<sup>3</sup>Institute for Clinical Molecular Biology, Christian-Albrechts-University Kiel, D-24105 Kiel, Germany

<sup>4</sup>Division of Gastroenterology, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115

<sup>5</sup>Inflammatory Bowel and Immunobiology Research Institute, Cedars-Sinai Medical Center, Los Angeles, CA 90048

<sup>6</sup>Department of Gastroenterology and Hepatology, Erasmus University Medical Center Rotterdam, 3015 CE Rotterdam, Netherlands

<sup>7</sup>Laboratory of Molecular and Cell Genetics, Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara 630-0192, Japan

<sup>8</sup>Division of Cell Medicine, Department of Life Science, Medical Research Institute, Kanazawa Medical University, Kahoku, Ishikawa 920-0293, Japan

<sup>9</sup>Eastman Dental Institute, University College London, London WC1E 6BT, England, UK

**ATG16L1<sup>T300A</sup>**, a major risk polymorphism in Crohn's disease (CD), causes impaired autophagy, but it has remained unclear how this predisposes to CD. In this study, we report that mice with *Atg16l1* deletion in intestinal epithelial cells (IECs) spontaneously develop transmural ileitis phenocopying ileal CD in an age-dependent manner, driven by the endoplasmic reticulum (ER) stress sensor IRE1 $\alpha$ . IRE1 $\alpha$  accumulates in Paneth cells of *Atg16l1<sup>ΔIEC</sup>* mice, and humans homozygous for *ATG16L1<sup>T300A</sup>* exhibit a corresponding increase of IRE1 $\alpha$  in intestinal epithelial crypts. In contrast to a protective role of the IRE1 $\beta$  isoform, hyperactivated IRE1 $\alpha$  also drives a similar ileitis developing earlier in life in *Atg16l1;Xbp1<sup>ΔIEC</sup>* mice, in which ER stress is induced by deletion of the unfolded protein response transcription factor XBP1. The selective autophagy receptor optineurin interacts with IRE1 $\alpha$ , and optineurin deficiency amplifies IRE1 $\alpha$  levels during ER stress. Furthermore, although dysbiosis of the ileal microbiota is present in *Atg16l1;Xbp1<sup>ΔIEC</sup>* mice as predicted from impaired Paneth cell antimicrobial function, such structural alteration of the microbiota does not trigger ileitis but, rather, aggravates dextran sodium sulfate-induced colitis. Hence, we conclude that defective autophagy in IECs may predispose to CD ileitis via impaired clearance of IRE1 $\alpha$  aggregates during ER stress at this site.

## INTRODUCTION

Crohn's disease (CD), a remitting-relapsing incurable chronic inflammatory bowel disease (IBD) commonly manifesting in early adulthood, has become a global health issue with rapidly increasing incidence in Asia and other parts of the world, fol-

lowing a steep rise in Europe and North America over the last decades (Molodecky et al., 2012). Although the environmental triggers remain enigmatic, association studies have successfully identified the genomic susceptibility landscape of CD (Jostins et al., 2012; Liu et al., 2015). With ~200 IBD risk loci scattered across the genome, which are individually mostly associated with minimal incremental risk (Jostins et al., 2012; Liu et al., 2015), it is easily overlooked that a huge proportion of overall explained heritability resides within very few risk genes, which are indeed specific for CD and not shared with other immune-related diseases. Among those, the coding polymorphism *ATG16L1<sup>T300A</sup>* is particularly noteworthy (Hampe et al., 2007; Rioux et al., 2007) as it has led to the

\*T.E. Adolph, J.W. Ashcroft, L. Niederreiter, and R. Bharti contributed equally to this paper.

\*\*R.S. Blumberg and A. Kaser contributed equally to this paper.

Correspondence to Arthur Kaser: ak729@cam.ac.uk; or Richard S. Blumberg: rblumberg@bwh.harvard.edu

T.E. Adolph's and L. Niederreiter's present address is Dept. of Medicine I, Gastroenterology, Endocrinology, and Metabolism, Medical University Innsbruck, 6020 Innsbruck, Austria.

Abbreviations used: CCA, canonical correlation analysis; CD, Crohn's disease; DSS, dextran sodium sulfate; H&E, hematoxylin and eosin; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; PAS, periodic acid-Schiff; qRT-PCR, quantitative RT-PCR; RIPa, radioimmunoprecipitation assay; SNP, single-nucleotide polymorphism; SPF, specific pathogen-free; TUNEL, TdT-mediated dUTP nick-end labeling; UPR, unfolded protein response.

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revelation that impaired autophagy (Murthy et al., 2014), and pathways that impinge upon it (Adolph et al., 2013), may be a main pathogenic disease mechanism in CD (Maloy and Powrie, 2011; Baxt and Xavier, 2015). This view is corroborated by additional risk genes involved in autophagy (e.g., *IRGM* and *LRRK2*) and by the discovery that *ATG16L1* and *NOD2*, the latter encoded by the CD-specific risk gene with the largest individual effect size, can interact during autophagosome formation (Cooney et al., 2010; Homer et al., 2010; Travassos et al., 2010). Although CD can arise anywhere in the gastrointestinal tract, both *NOD2* and *ATG16L1* risk variants are most strongly associated with CD involving the ileum (Prescott et al., 2007).

Abnormalities in the secretory compartment of Paneth cells, specialized intestinal epithelial cells (IECs) located at the base of crypts of the small intestine (Clevers and Bevins, 2013), are a common consequence of hypomorphic *ATG16L1* (Cadwell et al., 2008, 2010; Adolph et al., 2013; Deuring et al., 2014), *NOD2* (Kobayashi et al., 2005; Wehkamp et al., 2005), *IRGM* (Liu et al., 2013), and *LRRK2* (Zhang et al., 2015). In addition to disordered secretory granules that contain antimicrobial peptides and protein, CD patients and healthy individuals homozygous at the *ATG16L1*<sup>T300A</sup> single-nucleotide polymorphism (SNP) characteristically exhibit ER stress in their Paneth cells as indicated by high GRP78 expression (Deuring et al., 2014), which is also observed in mice with IEC-specific deletion of *Atg16l1* (Adolph et al., 2013). ER stress in Paneth cells, elicited via deletion of the unfolded protein response (UPR) transcription factor XBP1 in all IECs or Paneth cells specifically, induces a mild, superficial form of enteritis (Kaser et al., 2008; Adolph et al., 2013). In *Xbp1*<sup>ΔIEC</sup> mice, *ATG16L1*-dependent autophagy can partially compensate via yet unknown mechanisms for ER stress. Importantly, in the absence of such compensation, *Atg16l1*; *Xbp1*<sup>ΔIEC</sup> mice develop discontinuous, transmural fissuring ileitis phenocopying CD (Adolph et al., 2013). Here, we investigated the mechanisms engaged by impaired *ATG16L1*-dependent autophagy that drive ileal inflammation.

## RESULTS

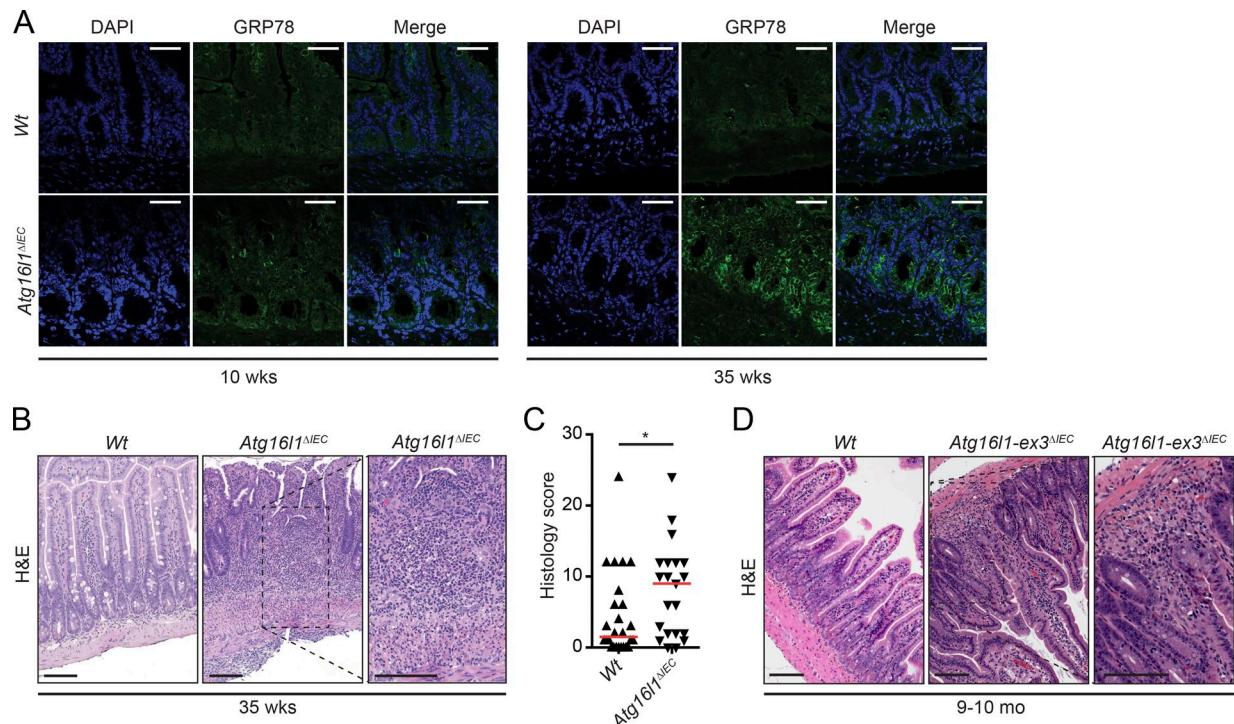
### Age-dependent manifestation of CD-like ileitis in *Atg16l1*<sup>ΔIEC</sup> mice

In nematodes, the capacity to resolve ER stress declines rapidly with increasing age (Taylor and Dillin, 2013). In addition, *Atg16l1*<sup>ΔIEC</sup> mice (Adolph et al., 2013) and humans homozygous for *ATG16L1*<sup>T300A</sup> (Deuring et al., 2014) exhibit increased ER stress in the intestinal epithelium. Therefore, we hypothesized that IECs, which are continuously exposed to a large variety of potential stressors that could have direct and indirect effects on protein folding, would become increasingly dependent on autophagy as a compensatory mechanism to resolve ER stress over time. Indeed, 35-wk-old *Atg16l1*<sup>ΔIEC</sup> mice exhibited higher expression of the ER stress marker GRP78 in crypt IECs compared with 10-wk-old *Atg16l1*<sup>ΔIEC</sup> mice, whereas *Wt* mice of either age exhibited

minimal GRP78 immunoreactivity (Fig. 1 A). Surprisingly, in light of earlier studies that *Atg16l1*<sup>ΔIEC</sup> and *Atg16l1*<sup>HM</sup> mice do not develop intestinal inflammation (Cadwell et al., 2008, 2010; Adolph et al., 2013; Conway et al., 2013), 35-wk-old *Atg16l1*<sup>ΔIEC</sup> mice developed a transmural ileitis, characterized by patchy infiltration of mononuclear and polymorphonuclear cells that penetrated the mucosa, submucosa, and muscularis propria of the ileum (Fig. 1, B and C). Other parts of the small intestine or the colon were not affected (not depicted). The features and severity of inflammation in 35-wk-old *Atg16l1*<sup>ΔIEC</sup> mice were indistinguishable from the CD-like ileitis that develops earlier in life in *Atg16l1*; *Xbp1*<sup>ΔIEC</sup> mice (Adolph et al., 2013), in which the UPR transcription factor XBP1 is co-deleted to induce ER stress (Kaser et al., 2008). Notably, transmural ileitis was also observed in 9–10-mo-old mice with an IEC-specific deletion of a genetically distinct *Atg16l1*-mutant allele (loxP sites flanking exon 3 rather than exon 1 [Adolph et al., 2013]) and held in a different (Los Angeles rather than Cambridge) animal facility (Fig. 1 D). These studies suggest that environmental and/or microbial stress accumulating over time can serve as a means to uncover the phenotypic consequences of impaired autophagy in the development of CD-like ileitis.

### IRE1 $\alpha$ promotes and IRE1 $\beta$ protects from CD-like ileitis in *Atg16l1*; *Xbp1*<sup>ΔIEC</sup> mice

To mechanistically examine the development of ileitis in the setting of impaired autophagy in the intestinal epithelium, we first focused our attention on 10-wk-old *Atg16l1*; *Xbp1*<sup>ΔIEC</sup> mice (Adolph et al., 2013) to allow for a tractable system to study. We had previously noted on immunoprecipitation and immunoblot experiments that total IEC lysates of *Atg16l1*; *Xbp1*<sup>ΔIEC</sup> mice exhibit profoundly increased IRE1 $\alpha$  expression and phosphorylation compared with lysates from *Xbp1*<sup>ΔIEC</sup> mice (Adolph et al., 2013), but it has remained unclear whether this has a role in transmural ileitis. IRE1 $\alpha$  is the ER transmembrane receptor that responds to protein misfolding by activating the XBP1 branch of the UPR, aimed at resolving such ER stress (Tirasophon et al., 2000; Lee et al., 2002; Hetz et al., 2011; Walter and Ron, 2011; Wang and Kaufman, 2016). However, increasing and prolonged ER stress causes IRE1 $\alpha$  activation to transition from an adaptive to a maladaptive, pro-death and proinflammatory UPR, associated with IRE1 $\alpha$  forming homodimers and large clusters, respectively (Li et al., 2010; Korennykh and Walter, 2012; Ghosh et al., 2014). Whereas IRE1 $\alpha$  was barely detectable in *Vil-Cre*<sup>neg</sup> (*Wt*) littermate controls, IEC-specific deletion of *Atg16l1* or *Xbp1* in 10-wk-old *Atg16l1*<sup>ΔIEC</sup> and *Xbp1*<sup>ΔIEC</sup> mice, respectively, led to increased IRE1 $\alpha$  immunoreactivity specifically in small intestinal crypts, with the highest levels detected when both *Atg16l1* and *Xbp1* were absent as observed in *Atg16l1*; *Xbp1*<sup>ΔIEC</sup> mice (Fig. 2 A). Furthermore, IRE1 $\alpha$  immunofluorescence localized most predominantly to Paneth cells, which were identified by antilysozyme and by lectin *Ulex europaeus* agglutinin 1 (UEA-1) containing (Fig. 2, B



**Figure 1. 35-wk-old *Atg16l1*<sup>ΔIEC</sup> mice exhibit increased ER stress and transmural CD-like ileitis.** (A) Representative images of GRP78 (green) immunoreactivity in 10- and 35-wk-old *Atg16l1*<sup>ΔIEC</sup> mice.  $n = 3$ . DAPI is shown in blue. Bars, 50  $\mu$ m. (B and C) Representative H&E images (B) and enteritis histology score of 35-wk-old *Atg16l1*<sup>ΔIEC</sup> mice maintained in a mouse norovirus-free facility (Cambridge; C).  $n = 25/21$ . The median is shown. \* $P < 0.05$ , Mann-Whitney  $U$  test. Note the transmural character of inflammation in *Atg16l1*<sup>ΔIEC</sup> mice compared with *Wt* mice. Bars, 100  $\mu$ m. (D) Representative H&E images of 9–10-mo-old *Atg16l1*-ex3<sup>ΔIEC</sup> mice generated and housed at Cedars-Sinai SPF mouse facility in Los Angeles. The inset shows the transmural character of the inflammation seen in *Atg16l1*-ex3<sup>ΔIEC</sup> mice.  $n = 10$ . Bars, 100  $\mu$ m.

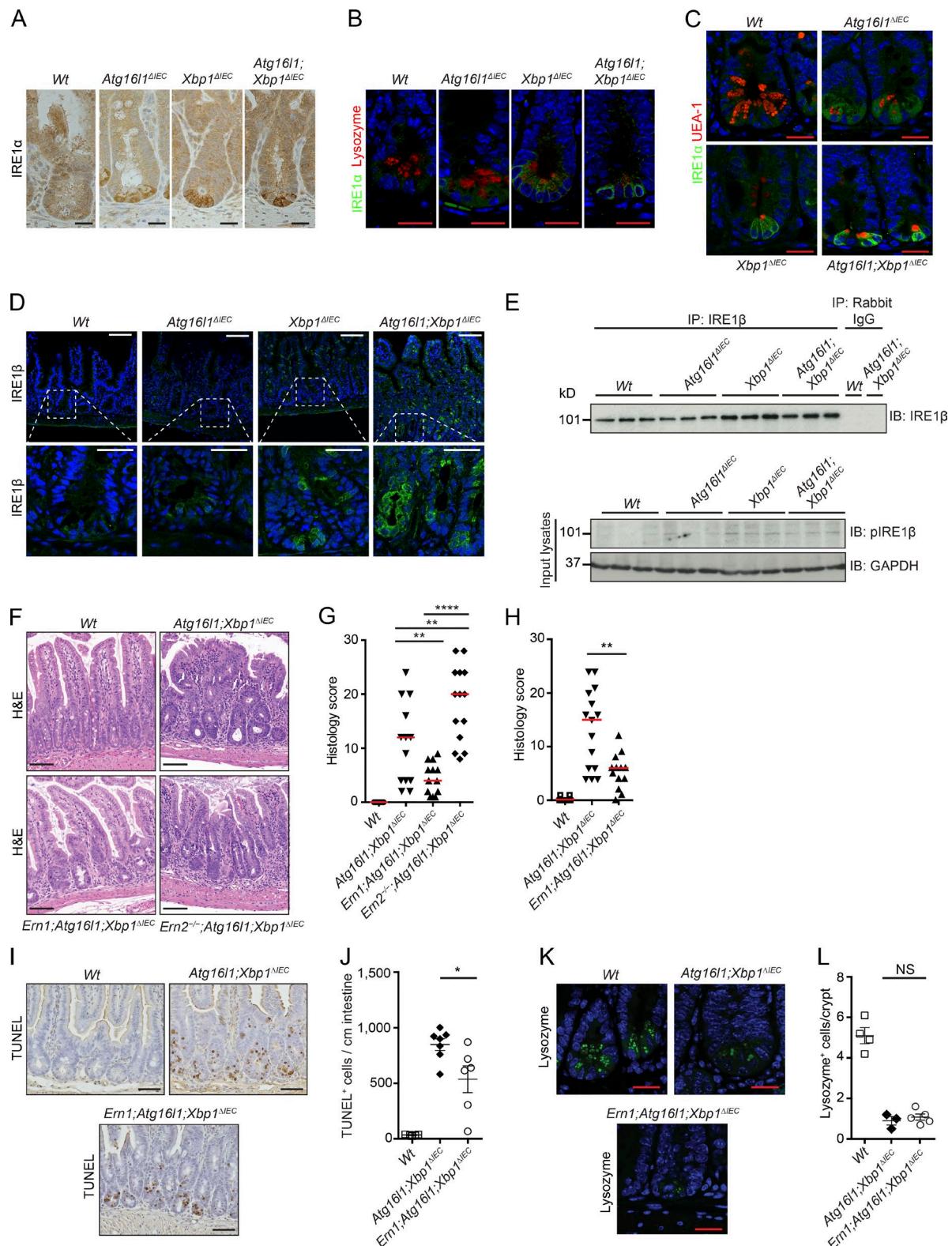
and C). Other highly secretory IECs such as goblet cells, where protein misfolding might be prevalent, did not exhibit increased IRE1 $\alpha$  immunoreactivity upon *Atg16l1* and/or *Xbp1* deletion (Fig. 2, A–C). Whereas IRE1 $\alpha$  (encoded by *Ern1*) at low levels is ubiquitously expressed, the intestinal and bronchial epithelium expresses a parologue with high sequence similarity, IRE1 $\beta$  (encoded by *Ern2*; Bertolotti et al., 2001; Iwawaki et al., 2001; Martino et al., 2013), reported to be involved in mucin 2 (MUC2) processing, goblet cell maturation (Tsuru et al., 2013), and enterocyte lipid metabolism (Iqbal et al., 2008). We noted increased immunoreactivity of IRE1 $\beta$  mostly in crypt but also in villus IECs of *Xbp1*<sup>ΔIEC</sup> and *Atg16l1*; *Xbp1*<sup>ΔIEC</sup> but not in *Atg16l1*<sup>ΔIEC</sup> mice at 10 wk of age when compared with *Wt* littermates (Fig. 2 D), which was corroborated by immunoprecipitation experiments from purified intestinal crypts (Fig. 2 E).

To investigate a possible contribution of IRE1 $\alpha$  and IRE1 $\beta$  to CD-like ileitis in *Atg16l1*; *Xbp1*<sup>ΔIEC</sup> mice, we generated *Ern1*; *Atg16l1*; *Xbp1*<sup>ΔIEC</sup> and *Ern2*<sup>−/−</sup>; *Atg16l1*; *Xbp1*<sup>ΔIEC</sup> mice, respectively. Fig. 2 (F and G) demonstrates that ileitis is prevented by IEC-specific deletion of *Ern1*, whereas germline deletion of *Ern2* increases the severity of inflammation. The effect of *Ern1* deletion was not specific for the particular microbial environment in this specific pathogen-free (SPF)

facility (Cambridge) but similarly observed in another SPF facility (Innsbruck), from which the *Atg16l1*; *Xbp1*<sup>ΔIEC</sup> parent strain has been rederived (Fig. 2 H). Protection from ileitis afforded by IEC-specific elimination of IRE1 $\alpha$  function in *Ern1*; *Atg16l1*; *Xbp1*<sup>ΔIEC</sup> mice was also associated with a decrease in the number of dying TdT-mediated dUTP nick-end labeling (TUNEL)<sup>+</sup> IECs (Fig. 2, I and J) but, importantly, not with rescue of impaired lysozyme staining in Paneth cells compared with *Atg16l1*; *Xbp1*<sup>ΔIEC</sup> mice (Fig. 2, K and L). Altogether, these experiments identified unexpected divergent roles of the two closely related IRE1 isoforms in the intestinal epithelium, with IRE1 $\alpha$  as driver of CD-like ileitis and IRE1 $\beta$  providing a protective function.

#### IRE1 $\alpha$ drives CD-like ileitis in 35-wk-old *Atg16l1*<sup>ΔIEC</sup> mice

Absence of *Xbp1* function abolishes cells' capacity for effective proteostasis causing ER stress, as observed in *Xbp1*<sup>ΔIEC</sup> mice, in turn triggering profound IRE1 $\alpha$  activation (Kaser et al., 2008). This accounts for the mild, superficial enteritis that develops in *Xbp1*<sup>ΔIEC</sup> mice (Adolph et al., 2013). The observation that IRE1 $\alpha$  accumulates most profoundly in *Atg16l1*; *Xbp1*<sup>ΔIEC</sup> Paneth cells and drives the transmural CD-like ileitis in *Atg16l1*; *Xbp1*<sup>ΔIEC</sup> mice suggested that autophagy may be directly involved in regulating IRE1 $\alpha$  function.



**Figure 2. IRE1 $\alpha$  controls CD-like inflammation.** (A) Representative images of IRE1 $\alpha$  immunohistochemistry (brown) on ileal sections of 10-wk-old Atg16l1 $^{\Delta IE}$ , Xbp1 $^{\Delta IE}$ , and Atg16l1;Xbp1 $^{\Delta IE}$  mice.  $n = 3$ . Hematoxylin is in blue. Bars, 20  $\mu$ m. (B) Representative confocal images of IRE1 $\alpha$  immunofluorescence (green) and Paneth cell-derived lysozyme (red) on ileal sections from the indicated genotypes at 10 wk of age. Note the perinuclear cytoplasmic IRE1 $\alpha$  immunoreactivity of Paneth cells from Atg16l1 $^{\Delta IE}$  mice that are lysozyme positive on the luminally oriented apex.  $n = 3$ . DAPI is in blue. Bars, 20  $\mu$ m.

However, it remained possible that decreased autophagy function and overactivation of IRE1 $\alpha$  caused by *Xbp1* deficiency are separate mechanisms and that ileitis is a combinatorial consequence. The spontaneous development of transmural ileitis phenocopying CD in 35-wk-old *Atg16l1 $^{\Delta IEC}$*  mice (Fig. 1, B and C) provided us an opportunity to test the hypothesis that IRE1 $\alpha$  activity is directly dependent on autophagy function. IRE1 $\alpha$  immunofluorescence at the small intestinal crypt bottom was indeed observed to increase from age 10–35 wk in *Atg16l1 $^{\Delta IEC}$*  mice, which contrasts with equally minimal staining in *Wt* littermates of either age (Fig. 3 A). Therefore, we generated *Ern1;Atg16l1 $^{\Delta IEC}$*  mice and studied them at 35 wk of age. In marked contrast to *Atg16l1 $^{\Delta IEC}$*  mice of the same age, 35-wk-old *Ern1;Atg16l1 $^{\Delta IEC}$*  mice housed in the same animal room were indeed protected from developing ileitis (Fig. 3, B and C). These results demonstrate that ATG16L1-dependent autophagy is involved in controlling IRE1 $\alpha$  activity and that IRE1 $\alpha$  drives inflammation in this mouse model of CD-like ileitis. Thus, decreased ATG16L1 function in IECs without genetic manipulation of UPR effectors such as XBP1 results in IRE1 $\alpha$ -dependent spontaneous ileitis.

#### IRE1 $\alpha$ accumulates in crypts of individuals homozygous for ATG16L1 $^{T300A}$

The *ATG16L1 $^{T300A}$*  polymorphism is common in populations of European ancestry, with a risk allele (G) frequency at *rs2241880* of 51 and 57% in healthy individuals and CD patients, respectively (Prescott et al., 2007). Based on the identification of IRE1 $\alpha$  as driver of CD-like ileitis in 35-wk-old *Atg16l1 $^{\Delta IEC}$*  mice, we assessed IRE1 $\alpha$  expression in noninflamed areas of the ileum in CD patients and healthy subjects. Fig. 3 D shows evidence for a granular staining pattern that is localized to the base of intestinal crypts in CD patients and healthy individuals who are homozygous for the risk allele (G/G). Quantitative immunofluorescence in  $n = 74$  healthy individuals and CD patients stratified according to *rs2241880* genotype revealed  $49.18 \pm 7.6\%$  of crypts positive for IRE1 $\alpha$  staining in individuals with G/G, compared with  $19.4 \pm 3.7\%$  and  $23.19 \pm 6.25\%$  in A/G and A/A genotypes, respectively (Fig. 3 E and Table S1). This suggests that ER stress–inducing noxae impinging on the mucosa of individuals homozygous for *ATG16L1 $^{T300A}$*  may trigger an exaggerated IRE1 $\alpha$  response.

(C) Representative confocal images of IRE1 $\alpha$  immunofluorescence (green) and the Paneth and goblet cell-type-specific lectin UEA-1 (red) on ileal sections from the indicated genotypes at 10 wk of age.  $n = 3$ . DAPI is in blue. Bars, 20  $\mu$ m. (D) Representative confocal images of IRE1 $\beta$  immunofluorescence (green) of the indicated genotypes at 10 wk of age. Note the specific IRE1 $\beta$  immunoreactivity of crypts (insets, bottom) and villus IECs from *Atg16l1;Xbp1 $^{\Delta IEC}$*  and *Xbp1 $^{\Delta IEC}$*  mice. DAPI is in blue.  $n = 3$ . Bars: (top) 50  $\mu$ m; (bottom) 20  $\mu$ m. (E) IRE1 $\beta$  immunoprecipitation (IP) and immunoblot (IB) as well as pIRE1 $\beta$  immunoblot in crypt lysates.  $n = 3$ . GAPDH was used as the loading control. (F and G) Representative H&E images (F) and their histological score (G) of 10-wk-old mice.  $n = 28/13/13/9$ . The median is shown. One-way ANOVA with Bonferroni's correction was used. Bars, 100  $\mu$ m. (H) Enteritis histology score of the indicated genotypes housed at the mouse norovirus–positive SPF animal facility (ZVTA) of the Medical University of Innsbruck.  $n = 12/15/13$ . The median is shown. Unpaired two-tailed Student's *t* test was used. (I and J) Representative images of TUNEL-labeled IECs (brown; I) and quantification (J).  $n = 5/7/6$ . The mean  $\pm$  SEM is shown. Mann-Whitney *U* test was used. Hematoxylin is in blue. Bars, 50  $\mu$ m. (K and L) Representative confocal images of lysozyme immunofluorescence in crypt IECs (green; K) and quantification (L).  $n = 4/3/5$ . The mean  $\pm$  SEM is shown. A Mann-Whitney *U* test was used. DAPI is in blue. Bars, 20  $\mu$ m. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*\*,  $P < 0.0001$ .

Altogether, these data establish a critical role of IRE1 $\alpha$  in driving intestinal inflammation consequent to impaired autophagy in the intestinal epithelium and Paneth cells in particular. The experiments that led to this conclusion pose several important questions: first, how do IRE1 $\alpha$  and IRE1 $\beta$  relate to each other within the intestinal epithelium and contribute to mucosal homeostasis? Second, how does IRE1 $\alpha$  accumulate in IECs of mice and humans with hypomorphic ATG16L1 function? Third, what is the contribution of the microbiota to triggering the spontaneous ileitis observed?

#### IRE1 $\alpha$ and IRE1 $\beta$ are cross-compensatory in the intestinal epithelium

IRE1 $\alpha$  and IRE1 $\beta$  are ER transmembrane receptors, which form homodimers in the presence of misfolded proteins, triggering their kinase and endoribonuclease function (Walter and Ron, 2011). IRE1 $\alpha$ 's endoribonuclease primarily splices *Xbp1* mRNA, causing a frame shift that converts XBP1 into the active transcription factor, whereas IRE1 $\beta$  predominantly engages in regulated IRE1-dependent decay that degrades specific (m)RNAs on the ER membrane (Hetz et al., 2011). Active IRE1 $\alpha$  (and presumably IRE1 $\beta$ ) forms a scaffold and recruits adapters and signaling molecules (Wochlbier and Hetz, 2011), and upon unabated, unresolvable stress, IRE1 $\alpha$  homodimers form large clusters that ultimately trigger cell death (Korennykh and Walter, 2012; Chen and Brandizzi, 2013; Cho et al., 2013; Ghosh et al., 2014; Maurel et al., 2014). The interrelationship between IRE1 $\alpha$  and IRE1 $\beta$  has not yet been investigated *in vivo*, despite their unique coexistence at mucosal surfaces that hints at a particular importance within this locale.

We indeed observed increased staining for IRE1 $\alpha$  at the crypt base in *Ern2 $^{-/-}$*  and conversely for IRE1 $\beta$  in *Ern1 $^{\Delta IEC}$*  mice (Fig. 4, A and B), whereas *Ern1* and *Ern2* mRNA expression remained unaltered under these conditions (Fig. 4, C and D), indicating posttranscriptional regulation. As expected, minimal baseline *Xbp1* splicing in *Wt* ileal crypt lysates was absent in *Ern1 $^{\Delta IEC}$*  and *Ern1 $^{\Delta IEC}$ ;Ern2 $^{-/-}$*  lysates (Fig. 4 E). Notably, *Xbp1* splicing was markedly increased in *Ern2 $^{-/-}$*  ileal crypt lysates (Fig. 4 E), indicating IRE1 $\alpha$  overactivation in the absence of IRE1 $\beta$  function. Deletion of the floxed exon 2 of *Xbp1* results in IRE1 $\alpha$  overactivation as observed through IRE1 $\alpha$  accumulation and phosphoryla-

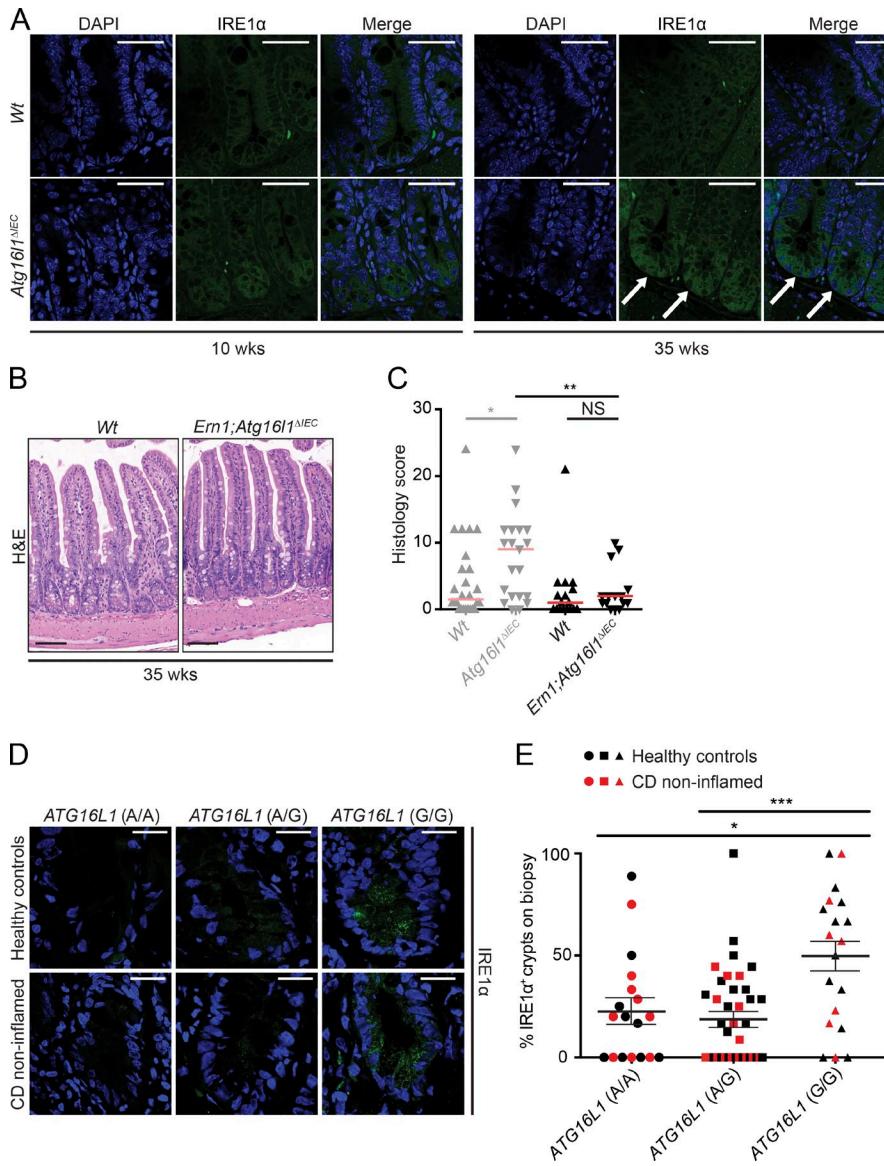


Figure 3. IRE1 $\alpha$  expression is increased in 35-wk-old *Atg16l1* $^{ΔIEC}$  mice and *ATG16L1* $^{T300A}$  patients and drives CD-like ileitis. (A) Representative confocal images of IRE1 $\alpha$  immunoreactivity (green; white arrows) in 10- and 35-wk-old *Atg16l1* $^{ΔIEC}$  mice.  $n = 3$ . DAPI is in blue. Bars, 50  $\mu$ m. (B) Representative H&E images of 35-wk-old *Ern1;Atg16l1* $^{ΔIEC}$  and *Wt* mice. Bars, 100  $\mu$ m. (C) Enteritis histology scores of 35-wk-old *Atg16l1* $^{ΔIEC}$  mice (the identical scores are depicted in Fig. 1 C) compared with 35-wk-old *Ern1;Atg16l1* $^{ΔIEC}$  mice and their respective *Wt* controls.  $n = 25/21/19/15$ . The median is shown. A Mann-Whitney  $U$  test was used. (D) Representative IRE1 $\alpha$  immunoreactivity (green) stratified by the *ATG16L1* risk allele (AA/AG/GG; A, healthy allele; G risk allele) in healthy controls ( $n = 9/22/12$ ) and noninflamed mucosa ( $n = 9/15/7$ ) of CD patients. DAPI is in blue. Bars, 20  $\mu$ m. (E) Quantification of IRE1 $\alpha^+$  crypts shown in D in healthy controls ( $n = 9/22/12$ ) and CD patients ( $n = 9/15/7$ ) according to their *ATG16L1* genotype ( $n = 18/37/19$ ). Mean  $\pm$  SEM is shown. One-way ANOVA with Bonferroni's correction was used. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

tion (Niederreiter et al., 2013) and massive splicing of *Xbp1* mRNA (the splicing site encoded by exon 4 is intact in the *Xbp1*<sup>ΔIREC</sup> allele; Fig. 4 E), as previously reported (Kaser et al., 2008; Niederreiter et al., 2013). *Ern1*;*Xbp1*<sup>ΔIREC</sup> ileal crypts exhibited some residual *Xbp1* splicing, which was completely abrogated in *Ern1*;*Xbp1*<sup>ΔIREC</sup>;*Ern2*<sup>-/-</sup> ileal crypts (Fig. 4 E), indicating that IRE1 $\beta$  may contribute to *Xbp1* splicing. Consistent with a cross-compensatory role of IRE1 $\alpha$  and IRE1 $\beta$ , *Ern1*<sup>ΔIREC</sup>;*Ern2*<sup>-/-</sup> Paneth cells exhibited a condensed ER and a nearly complete collapse of the secretory compartment on transmission electron microscopy (Fig. 4, F and G), reminiscent of *Xbp1*<sup>ΔIREC</sup> mice (Kaser et al., 2008), whereas single-mutant *Ern1*<sup>ΔIREC</sup> and *Ern2*<sup>-/-</sup> crypts were almost indistinguishable from *Wt* controls with only minimal distension of the ER (Fig. 4, F and G). *Ern1*<sup>ΔIREC</sup>;*Ern2*<sup>-/-</sup> mice exhibited an absence of lysozyme immunofluorescence reflecting the

massive alterations in the secretory compartment observed in comparison with *Ern1<sup>ΔIEC</sup>* and *Ern2<sup>-/-</sup>* mice, which exhibited only minimally decreased lysozyme staining compared with Wt controls (Fig. 4, H and I). Both periodic acid–Schiff (PAS; Fig. 5, A and B) and MUC2 (Fig. 5, C and D) staining for mucus-producing goblet cells was nearly completely absent in the small intestines of *Ern1<sup>ΔIEC</sup>;Ern2<sup>-/-</sup>* mice, with decreased MUC2 staining observed in *Ern2<sup>-/-</sup>* single-mutant mice (Fig. 5, C and D), consistent with an important role of IRE1 $\beta$  in mucin secretion (Tsuru et al., 2013). Whereas *Ern1<sup>ΔIEC</sup>;Ern2<sup>-/-</sup>* mice developed low levels of spontaneous superficial enteritis (Fig. 5, E and F) accompanied by epithelial hyperproliferation (Fig. 5, G and H) and increased epithelial cell death (Fig. 5, G and H), these changes were not observed in *Ern1<sup>ΔIEC</sup>* and *Ern2<sup>-/-</sup>* single-mutant mice (Fig. 5, E–J). Together, these data demonstrate that IRE1 $\alpha$  and IRE1 $\beta$

complement each other to maintain Paneth cell and intestinal homeostasis, although their roles fundamentally diverge in *Atg16l1;Xbp1<sup>ΔIEC</sup>* mice, with IRE1 $\alpha$  driving and IRE1 $\beta$  protecting from CD-like ileitis.

### IRE1 $\alpha$ accumulates in the absence of selective autophagy receptor optineurin

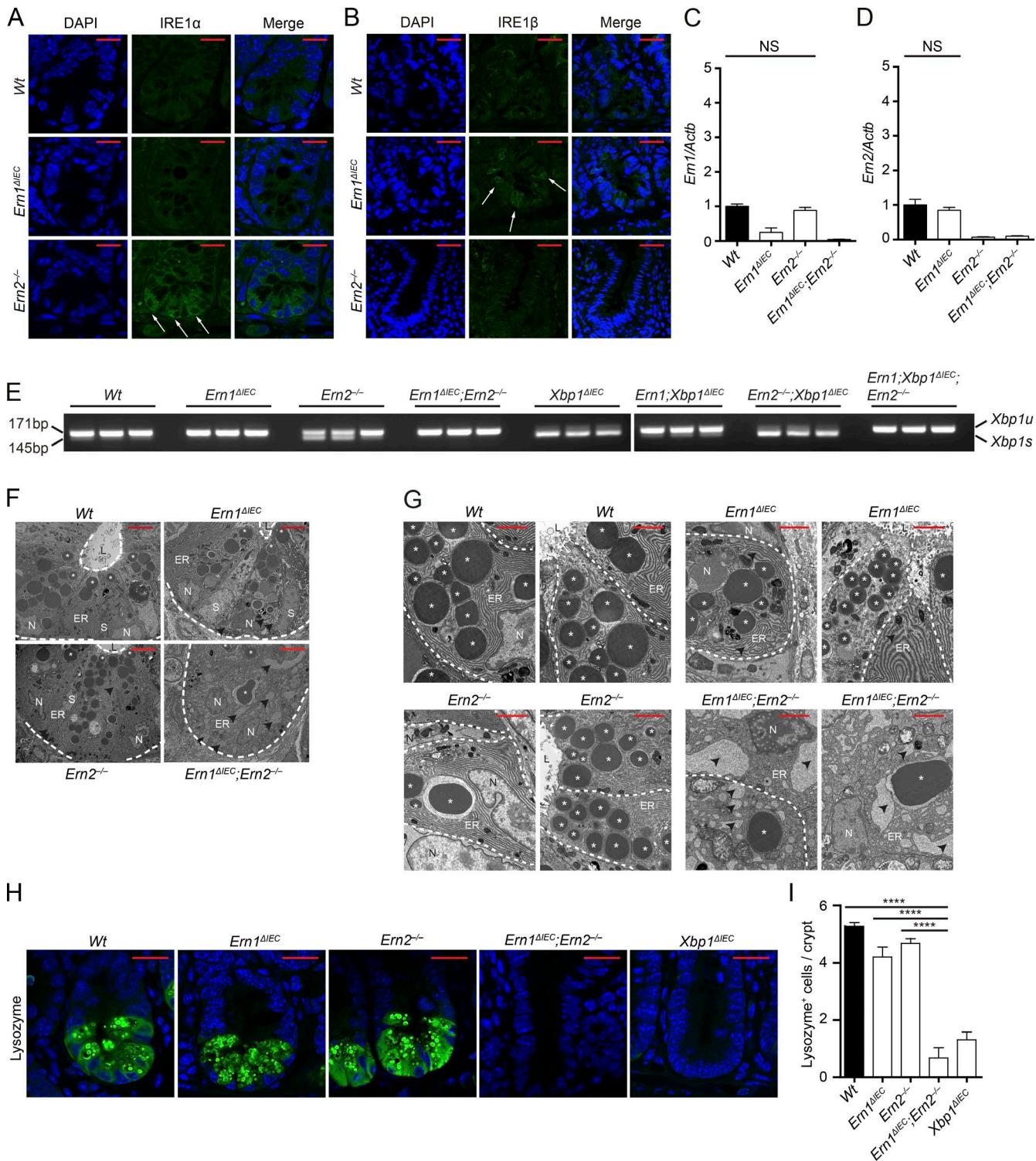
The observed increased IRE1 $\alpha$  activity in *Atg16l1<sup>ΔIEC</sup>*, *Xbp1<sup>ΔIEC</sup>*, and *Atg16l1;Xbp1<sup>ΔIEC</sup>* mice could not be explained by transcriptional regulation of *Ern1* (Fig. 6 A), suggesting that autophagy may be involved in IRE1 $\alpha$  protein turnover. Autophagy engulfs intracellular material, e.g., organelles (mitophagy, pexophagy, and ER-phagy), macromolecular complexes (aggrophagy), or infectious agents (xenophagy), and delivers it to lysosomes for degradation (Levine et al., 2011; Schuck et al., 2014; Khaminets et al., 2015). As reductionist model systems to study the involvement of autophagy in IRE1 $\alpha$  protein turnover under baseline and ER stress conditions, we chose the mouse small intestinal epithelial cell line MODE-K expressing control shRNA (MODE-K.*iCtrl*) and *Xbp1*-specific shRNA vectors (MODE-K.*iXbp1*; Kaser et al., 2008), respectively. Baflomycin A, an inhibitor of vacuolar H $^{+}$  ATPase that prevents acidification of autophagolysosomes and hence degradation of their cargo, increased IRE1 $\alpha$  protein levels in MODE-K.*iXbp1* cells, which experience ER stress, but not in MODE-K.*iCtrl* cells (Fig. 6 B). Conversely, augmentation of autophagy via the mechanistic target of rapamycin inhibitor rapamycin decreased IRE1 $\alpha$  protein levels in MODE-K.*iXbp1* but not MODE-K.*iCtrl* cells, whereas inhibition of protein translation via cycloheximide decreased IRE1 $\alpha$  levels in both cell lines (Fig. 6 C). This suggested that autophagy may contribute to IRE1 $\alpha$  turnover predominantly during ER stress.

Considering the formation of large clusters of IRE1 $\alpha$  during profound and sustained ER stress, we hypothesized that IRE1 $\alpha$  might be removed via a selective autophagy process under such conditions. Formerly considered an entirely nonspecific process (bulk [macro-] autophagy), selective autophagy processes have emerged that identify their cargo via specific receptors and adaptors in a ubiquitin-dependent and -independent fashion and deliver them to autophagosomes (Weidberg et al., 2011; Rogov et al., 2014; Stolz et al., 2014; Khaminets et al., 2016). Therefore, we studied the involvement of several known selective autophagy receptors, p62 (sequestosome-1 [*Sqstm1*]), NBR1 (neighbor of BRCA1 gene 1 [*Nbr1*]), optineurin (*Optn*), and NDP52 (*CALCOCO2*; Wild et al., 2011; Khaminets et al., 2016), with the latter two having previously been implicated in CD (Ellinghaus et al., 2013; Chew et al., 2015; Smith et al., 2015). Fig. 6 (D and E) demonstrates that optineurin and NBR1 reciprocally immunoprecipitated with IRE1 $\alpha$  in MODE-K.*iXbp1* lysates, whereas biochemical interaction with optineurin was minimal, but detectable, in MODE-K.*iCtrl* cells. p62 coimmunoprecipitated with IRE1 $\alpha$  at equal levels in MODE-K.*iCtrl* and MODE-K.*iXbp1* cells (Fig. 6 F). NDP52 coimmuno-

precipitated with IRE1 $\alpha$  and vice versa in the human colonic epithelial cell line HT29 under basal conditions (Fig. 6 G) as well as after pharmacological ER stress induction with tunicamycin (Fig. 6 H). Altogether, these data suggest that several selective autophagy receptors biochemically interact with IRE1 $\alpha$ . Importantly, silencing of *Optn* via siRNA resulted in increased IRE1 $\alpha$  protein levels in MODE-K.*iXbp1* cells and only a minimal increase in MODE-K.*iCtrl* cells (Fig. 6 I). In contrast to *Optn*, silencing of *Sqstm1* or *Nbr1* in MODE-K.*iXbp1* did not affect IRE1 $\alpha$  protein levels (Fig. 6 J), and co-silencing of these receptors in conjunction with *Optn* did not have synergistic effects (not depicted). Similarly, silencing of *NDP52* in HT29 cells did not increase IRE1 $\alpha$  protein levels at baseline or after pharmacological ER stress induction with tunicamycin (Fig. 6 K). Importantly, IRE1 $\alpha$  was observed to interact with optineurin in vivo, as demonstrated by detection of IRE1 $\alpha$  protein in anti-optineurin immunoprecipitates in small intestinal crypt lysates from *Xbp1<sup>ΔIEC</sup>* mice, whereas IRE1 $\alpha$  was only just above detection limit in immunoprecipitates from *Wt* crypt lysates (Fig. 6 L). Altogether, these data suggest that during profound or prolonged ER stress, IRE1 $\alpha$  may be recruited to autophagosomes via optineurin, followed by IRE1 $\alpha$ 's degradation, hence implying selective autophagy as a mechanism for terminating IRE1 $\alpha$  signaling during ER stress.

### Mice with CD-like ileitis harbor a dysbiotic microbiota

The spontaneous CD-like ileitis observed in *Atg16l1;Xbp1<sup>ΔIEC</sup>* mice also provided a unique opportunity to interrogate the contribution of alterations in the microbiota caused by secretory Paneth cell abnormalities to intestinal inflammation. Mice of the *Atg16l1;Xbp1* colony (*Atg16l1;Xbp1<sup>ΔIEC</sup>* co-housed with their *Vil-Cre<sup>neg</sup>* *Wt* [*Atg16l1<sup>fl/fl</sup>*; *Xbp1<sup>fl/fl</sup>*] littermates) exhibited an overall distinct tissue-adherent ileal microbial composition compared with *Atg16l1* (*Atg16l1<sup>ΔIEC</sup>* and their *Wt* [*Atg16l1<sup>fl/fl</sup>*] littermates) or *Xbp1* (*Xbp1<sup>ΔIEC</sup>* and *Wt* [*Xbp1<sup>fl/fl</sup>*] littermates) colonies (adonis; Bray-Curtis: genotype,  $R^2 = 0.14812$ ,  $P = 0.000999$ ; gender,  $R^2 = 0.07445$ ,  $P = 0.003996$ ; cage,  $R^2 = 0.38101$ ,  $P = 0.000999$ ; litter,  $R^2 = 0.02$ ,  $P = 0.99$ ; Jaccard: genotype,  $R^2 = 0.12052$ ,  $P = 0.000999$ ; gender,  $R^2 = 0.06340$ ,  $P = 0.004995$ ; cage,  $R^2 = 0.36132$ ,  $P = 0.000999$ ; litter,  $R^2 = 0.064$ ,  $P = 0.99$ ; Fig. 7, A–F), with the maximum microbial shift being observed between *Atg16l1;Xbp1<sup>ΔIEC</sup>* and *Atg16l1<sup>ΔIEC</sup>* mice (adonis; Bray-Curtis:  $R^2 = 0.22912$ ,  $P = 0.01598$ ; Jaccard:  $R^2 = 0.18951$ ,  $P = 0.01199$ ). Specifically, the *Atg16l1;Xbp1<sup>ΔIEC</sup>* mice exhibited an increased abundance of *Bacteroidetes* (Student's *t* test with Welch's correction,  $P = 0.0295$ ) and decreased *Firmicutes* (Student's *t* test with Welch's correction,  $P = 0.0288$ ) compared with *Atg16l1<sup>ΔIEC</sup>* mice (Fig. 7, A and B). Differences in bacterial composition were further highlighted by a relatively small overlap of operational taxonomic units between *Atg16l1<sup>ΔIEC</sup>*, *Xbp1<sup>ΔIEC</sup>*, and *Atg16l1;Xbp1<sup>ΔIEC</sup>* mice, respectively (not depicted), whereas species diversity and species richness were comparable between respective colonies.



**Figure 4. Cross-compensatory up-regulation of IRE1 $\alpha$  and IRE1 $\beta$  maintains Paneth cell homeostasis.** (A and B) Representative confocal images of IRE1 $\alpha$  (A) or IRE1 $\beta$  (B) immunofluorescence (green) of the indicated genotypes at 10 wk of age. Note the cross-compensatory up-regulation of IRE1 $\beta$  and IRE1 $\alpha$  in crypts of  $Em1^{\Delta IEC}$  and  $Em2^{-/-}$  mice, respectively (white arrows).  $n = 3$ . DAPI is in blue. Bars, 20  $\mu$ m. (C and D)  $Em1$  (C) and  $Em2$  (D) mRNA expression measured by qRT-PCR in ileal scrapings of the indicated genotypes normalized to  $Actb$  expression.  $n = 20/10/16/15$ . The mean  $\pm$  SEM is shown. One-way ANOVA with Bonferroni's correction was used. Note that IRE1 $\alpha$  and IRE1 $\beta$  (encoded by  $Em1$  and  $Em2$ ) in  $Em2^{-/-}$  and  $Em1^{\Delta IEC}$  mice, respectively, are not transcriptionally regulated. (E)  $Xbp1$  splicing in crypt IECs from the indicated genotypes indicative of upstream activation of IRE1 $\alpha$ /IRE1 $\beta$ . Note increased

(Fig. 7, G and H). *Wt* littermates of each colony appeared to acquire the respective knockout microbiota, as their microbial compositions were indistinguishable (adonis; Bray-Curtis; *Atg16l1* colony,  $R^2 = 0.05133$ ,  $P = 0.7672$ ; *Xbp1* colony,  $R^2 = 0.04037$ ,  $P = 0.59341$ ; *Atg16l1;Xbp1* colony,  $R^2 = 0.10241$ ,  $P = 0.14885$ ; Fig. 7, E and F).

Acquisition of an altered microbiota by *Wt* mice cohoused with their IEC-mutant littermates afforded an opportunity to dissect functional consequences via cross-fostering studies. *Wt* litters cross-fostered by *Atg16l1;Xbp1<sup>ΔIEC</sup>* mice indeed exhibited increased severity of colitis induced by dextran sodium sulfate (DSS), compared with *Wt* mice cross-fostered with *Wt* mice (Fig. 8, A–E). Importantly, all *Wt* litters and *Wt* foster dams were *V-Cre<sup>neg</sup>;Atg16l1<sup>fl/fl</sup>;Xbp1<sup>fl/fl</sup>* mice, hence stemming from the *Atg16l1;Xbp1<sup>ΔIEC</sup>* colony. Altogether, these data established that the *Atg16l1;Xbp1<sup>ΔIEC</sup>* microbiota is indeed dysbiotic and aggravates exogenously induced experimental colitis.

### Ileal dysbiosis does not trigger ileitis

*Wt* mice cohoused with their *Atg16l1;Xbp1<sup>ΔIEC</sup>* littermates did not develop any intestinal inflammation, nor did cohoused *Wt* littermates of *Xbp1<sup>ΔIEC</sup>* mice (Fig. 8 F), despite an almost identical ileal microbial ecology between *Wt* mice and their respective IEC-mutant littermates (Fig. 7, A and B). Having established the dysbiotic, aggravating effect of the *Atg16l1;Xbp1<sup>ΔIEC</sup>* microbiota on DSS colitis (Fig. 8, C–E) and the transferability of the microbiota between IEC-mutant mice, their *Wt* littermates, and cross-fostered mice (Fig. 7, A–F; and Fig. 8, C–E), we posed the question whether transferable microbial elements might aggravate ileitis in genetically susceptible *Atg16l1<sup>ΔIEC</sup>* or *Xbp1<sup>ΔIEC</sup>* mice. Cross-fostering of *Wt*, *Atg16l1<sup>ΔIEC</sup>*, and *Xbp1<sup>ΔIEC</sup>* litters with *Atg16l1;Xbp1<sup>ΔIEC</sup>* foster dams revealed that histology scores and features were indistinguishable from non-cross-fostered mice with the respective genotypes (Fig. 8, G and H). Hence, the genetically susceptible *Atg16l1<sup>ΔIEC</sup>* and *Xbp1<sup>ΔIEC</sup>* litters cross-fostered with *Atg16l1;Xbp1<sup>ΔIEC</sup>* foster dams did not develop CD-like ileitis as observed in *Atg16l1;Xbp1<sup>ΔIEC</sup>* mice or 35-wk-old *Atg16l1<sup>ΔIEC</sup>* mice. Consistent with ileal dysbiosis not triggering ileal inflammation in this model, *Ern1;Atg16l1;Xbp1<sup>ΔIEC</sup>* mice, which were protected from ileitis (Fig. 2, F and G; and Fig. 9 A), exhibited an ileal microbiota similar to *Atg16l1;Xbp1<sup>ΔIEC</sup>* mice (Fig. 9, B–E) and Paneth cells that remained profoundly impaired in ly-

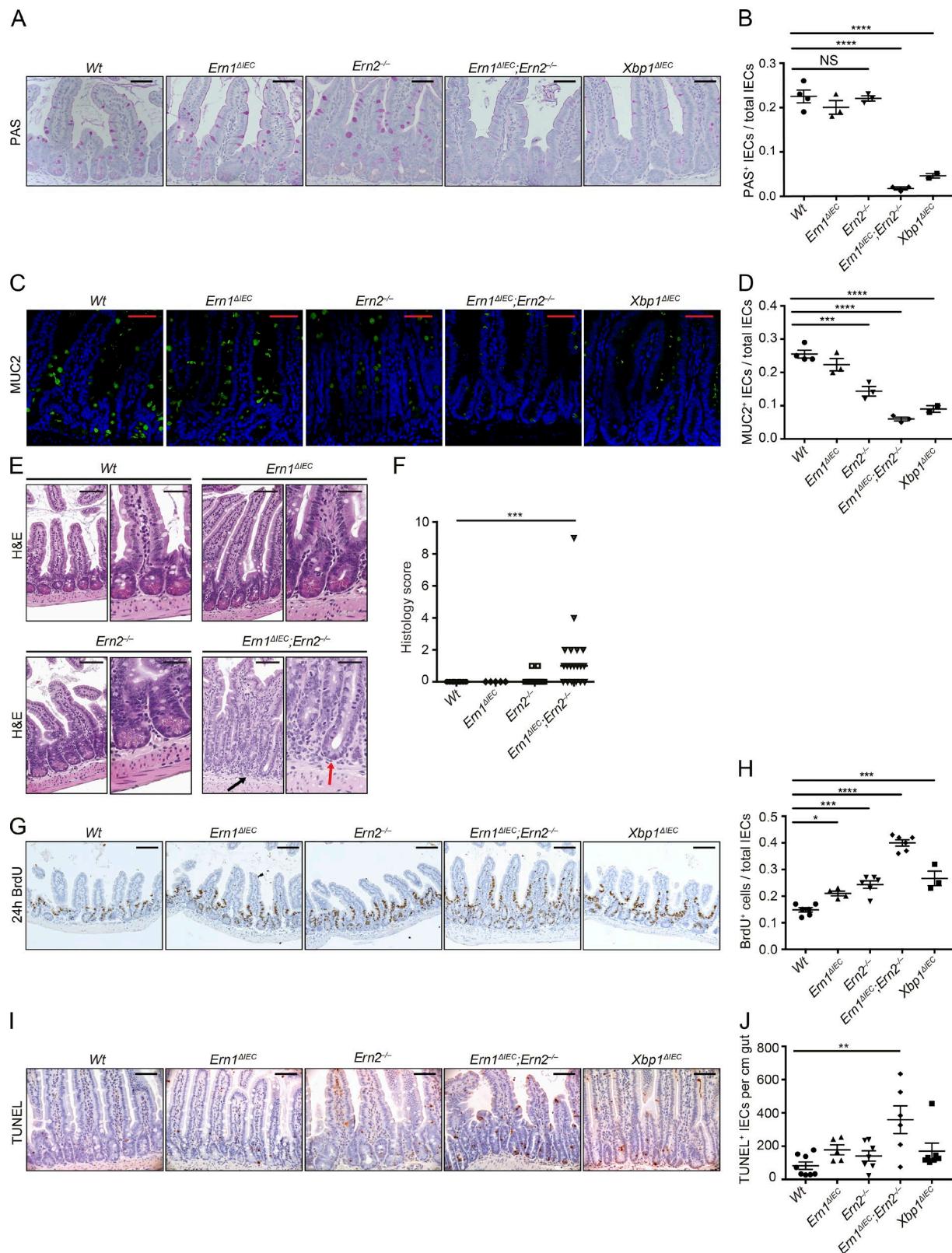
sozyme staining (Fig. 2, K and L). Specifically,  $\beta$  diversity did not show any differences between *Ern1;Atg16l1;Xbp1* and *Atg16l1;Xbp1* colonies (adonis; Bray-Curtis; genotype,  $R^2 = 0.08782$ ,  $P = 0.08192$ ; gender,  $R^2 = 0.01233$ ,  $P = 0.90809$ ; litter,  $R^2 = 0.10755$ ,  $P = 0.74126$ ; Fig. 9 D). Consistently, only marginal significance between both colonies was found in canonical correlation analysis (CCA; genotype,  $F = 1.7166$ ,  $P = 0.045$ ; gender,  $F = 0.6475$ ,  $P = 0.785$ ; Fig. 9 E). Moreover, no differences were detected in a pairwise comparison between *Wt* and littermate IEC-mutant mice of both colonies (adonis; Bray-Curtis; *Atg16l1;Xbp1* colony,  $R^2 = 0.7456$ ,  $P = 0.435$ ; *Ern1;Atg16l1;Xbp1* colony,  $R^2 = 1.56$ ,  $P = 0.178$ ; Fig. 9 D). The results demonstrate that IRE1 $\alpha$ -dependent development of ileitis is remarkably independent of the dysbiosis present in *Atg16l1;Xbp1<sup>ΔIEC</sup>* mice.

### DISCUSSION

We report IRE1 $\alpha$  as the main driver of spontaneous ileitis phenocopying CD that develops with increasing age in mice mutant for *Atg16l1* in the intestinal epithelium. Exaggerated UPR signaling through IRE1 $\alpha$  caused by impairment of a selective autophagy process may be an important mechanism in how hypomorphic *ATG16L1<sup>T300A</sup>* contributes to disease in the context of yet unknown environmental triggers that cause ER stress. In contrast, alterations in ileal microbiota structure, although present in mice with CD-like ileitis, importantly did not trigger spontaneous ileal but aggravated chemically induced colonic inflammation.

Host genetic alterations can render the microbiota inflammation inducing, e.g., as observed for colitis in *Nod2* or *Nlrp6* mutants upon exposure to DSS (Elinav et al., 2011; Couturier-Maillard et al., 2013) or for ileitis in the *Tnf<sup>ΔARE</sup>* model (Schaubek et al., 2016). Because alterations in the secretory compartment, lysozyme, and  $\alpha$ -defensin secretion of Paneth cells have emerged as common features observed in carriers of CD-specific risk gene variants (Kobayashi et al., 2005; Wehkamp et al., 2005; Cadwell et al., 2008; VanDussen et al., 2014; Zhang et al., 2015) and microbial community structure differs between CD and healthy individuals (Frank et al., 2007; Gevers et al., 2014; Rehman et al., 2016), it has been conjectured that such host genetically induced dysbiosis might be critical for triggering intestinal inflammation in CD. Our data challenge this hypothesis by demonstrating that host mutant-induced alterations in the ileal microbiota are not sufficient to trigger or promote ileitis, although they

*Xbp1* splicing in *Ern2<sup>-/-</sup>* crypts indicative of upstream activation of IRE1 $\alpha$  (also see A), whereas IRE1 $\beta$  appears to have a minor function for *Xbp1* splicing in *Ern1;Xbp1<sup>ΔIEC</sup>* mice which is absent in *Ern1;Xbp1<sup>ΔIEC</sup>;Ern2<sup>-/-</sup>*.  $n = 3$ . (F) High magnification transmission electron microscopy micrographs of ileal crypts depicting Paneth cells from the indicated genotypes.  $n = 2$ . Bars, 5  $\mu$ m. Dashed lines denote crypt unit. (G) High magnification transmission electron microscopy micrographs of ileal crypts depicting Paneth cells from the indicated genotypes.  $n = 2$ . Bars, 2  $\mu$ m. Dashed lines denote single Paneth cells. (F and G) L, lumen; N, nucleus; S, stem cell. White asterisks label Paneth cell granules. Note the absence of intact Paneth cell granules and the dilated ER lumen (black arrowheads). (H and I) Representative confocal images of lysozyme immunoreactivity (green; H) and quantification of lysozyme $^+$  cells per crypt (I) on ileal sections of the indicated genotypes.  $n = 4/3/4/3/4$ . The mean  $\pm$  SEM is shown. One-way ANOVA with Bonferroni's correction was used.  $****$ ,  $P < 0.0001$ . DAPI is in blue. Bars, 20  $\mu$ m.



**Figure 5. Cross-compensation of IRE1 $\alpha$  and IRE1 $\beta$  maintains intestinal homeostasis.** (A and B) Representative PAS staining on ileal sections from the indicated genotypes at 10 wk of age (A) with quantification of PAS-labeled IECs per total IECs along the crypt villus axis (B). Note the absence of PAS<sup>+</sup> goblet cells in *Ern1 $^{\Delta IE}$ ;Ern2 $^{-/-}$*  mice.  $n = 4/3/3/3/2$ . The mean  $\pm$  SEM is shown. One-way ANOVA with Bonferroni's correction was used. Hematoxylin coun-

might increase severity of established colitis. Nonetheless, the presence of a microbiota is required for developing small intestinal inflammation because *Xbp1<sup>ΔIEC</sup>* mice rederived germ free are protected from enteritis (Adolph et al., 2013) and do not accumulate IRE1 $\alpha$  in Paneth cells (not depicted). It remains entirely possible that outside of an SPF environment, the altered microbiota might provide a favorable niche for potential pathogens, e.g., as present in human's real-life exposome, which may impact on the disease mechanism as environmental factors. Along these lines, host gene mutants have been reported to favor the outgrowth of specific pathogens that can cause colitis, such as *Helicobacter typhlonius* in TRUC (*Tbx21*<sup>-/-</sup>; *Rag2*<sup>-/-</sup> ulcerative colitis) mice (Garrett et al., 2007; Powell et al., 2012), or mutants may be susceptible to opportunistic pathogens, such as *Helicobacter hepaticus* in *Nod2*<sup>-/-</sup> mice (Biswas et al., 2010). Indeed, the pathotype adherent and invasive *E. coli*, which has been associated with ileal CD (Darfeuille-Michaud et al., 2004) and with granulomatous colitis in boxer dogs (Simpson et al., 2006), is restricted by ATG16L1-dependent autophagy in IEC lines (Lapaquette et al., 2010).

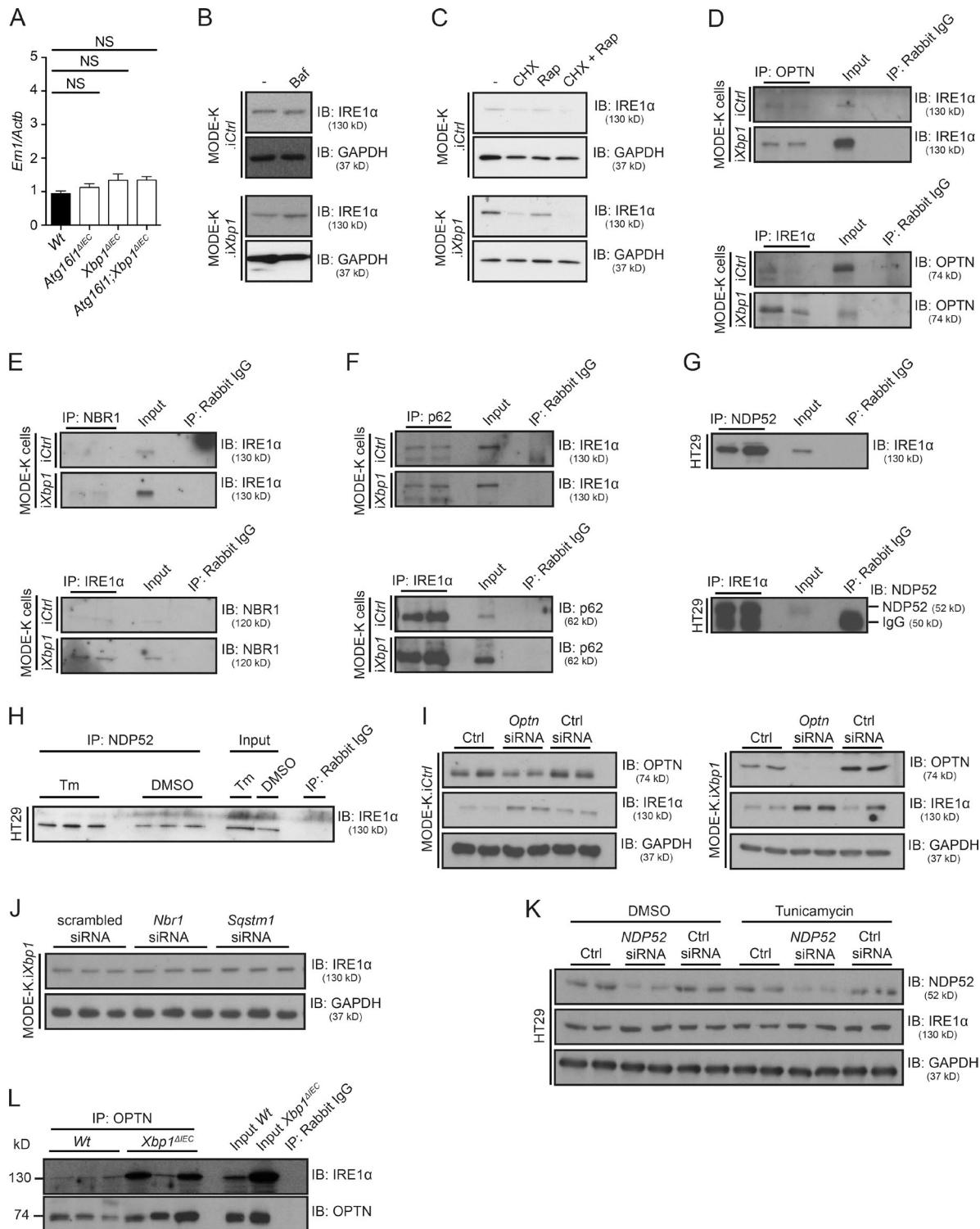
The development of ileitis with increasing lifetime in mice with a null variant of *Atg16l1* in IECs has exposed the important role of ATG16L1-dependent autophagy for restraining IRE1 $\alpha$  hyperactivation. Although rapid age-dependent decline in the ability to resolve ER stress has been observed in *Caenorhabditis elegans* (Taylor and Dillin, 2013), aging will likely play only a very subordinate, if any, role as a disease trigger in human *ATG16L1<sup>T300A</sup>* carriers; the high risk allele frequency of *ATG16L1<sup>T300A</sup>* in itself already argues against such a simple relationship (Jostins et al., 2012). The continual accumulation of stress hits over time that lead to IRE1 $\alpha$  engagement may at some point overwhelm the epithelium's capacity to resolve ER stress, whereby hypomorphic *ATG16L1<sup>T300A</sup>* may be an important determinant of that threshold. In fact, the ER serves as a central integrator of the state of the cell (Hotamisligil, 2010), and IECs and Paneth cells at the inner surface are particularly exposed to environmental factors that may impinge on this finely balanced system. Such factors may arise from, for example, infections (Pillich et al., 2012), microbiota constituents (Tashiro et al., 2007), toxins (Sun et al., 2014), or from components of the diet (Nezami et al., 2014; Stancu et al., 2015; Yin et al., 2015).

terstaining is in light blue. Bars, 50  $\mu$ m. (C and D) Representative confocal images of MUC2 immunoreactivity (green) on ileal sections from the indicated genotypes at 10 wk of age (C) with quantification of MUC2 $^+$  goblet cells per total IECs along the crypt villus axis (D). Note the absence of MUC2 $^+$  goblet cells in *Ern1<sup>ΔIEC</sup>;Ern2*<sup>-/-</sup> mice.  $n = 4/3/3/3/2$ . The mean  $\pm$  SEM is shown. One-way ANOVA with Bonferroni's correction was used. DAPI is in blue. Bars, 50  $\mu$ m. (E and F) Representative H&E images (E) of the indicated genotypes at 10 wk of age and their histological score (F).  $n = 10/5/17/19$ . The median is shown. A Mann-Whitney *U* test was used. Note the absence of eosinophilic Paneth cell granule (red arrow) and the surrounding basal infiltration of mononuclear cells in *Ern1<sup>ΔIEC</sup>;Ern2*<sup>-/-</sup> mice (black arrow). Bars: (left) 100  $\mu$ m; (right) 50  $\mu$ m. (G and H) Representative images of BrdU-labeled IECs (brown; G) from the indicated genotypes at 10 wk of age after 24 h of BrdU incorporation with quantification of BrdU $^+$  IECs per total IECs along the crypt villus axis (H).  $n = 6/3/4/6/6$ . The mean  $\pm$  SEM is shown. One-way ANOVA with Bonferroni's correction was used. Hematoxylin counterstaining is in blue. Bars, 100  $\mu$ m. (I and J) Representative images of TUNEL labeling (brown; I) and quantification of TUNEL $^+$  cells per cm gut on ileal sections (J) of the indicated genotypes at 10 wk of age.  $n = 8/7/5/7/6$ . The mean  $\pm$  SEM is shown. One-way ANOVA with Bonferroni's correction was used. Hematoxylin counterstaining is in blue. Bars, 50  $\mu$ m. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ .

Notably, high fat diet and obesity, the latter associated with markedly increased risk for developing CD (Khalili et al., 2015), have long been known to associate with ER stress in liver and adipose tissue (Hotamisligil, 2010) but also in Paneth cells (Hodin et al., 2011).

The principle mechanism that is activated by the UPR for removal of misfolded proteins, ER-associated degradation, remarkably also controls the turnover of IRE1 $\alpha$  via the Sel1L-Hrd1 ER-associated degradation complex under basal conditions (Sun et al., 2015). In contrast, Sel1L-dependent IRE1 $\alpha$  degradation is attenuated under conditions of ER stress (Sun et al., 2015), suggesting that optineurin-dependent selective autophagy may then become increasingly important as suggested by our data. As IRE1 $\alpha$  dimerization, oligomerization, and higher-order cluster formation are associated with escalating states of IRE1 $\alpha$  activation and a transition from homeostatic to pro-death and proinflammatory UPR (Korennyykh and Walter, 2012; Ghosh et al., 2014; Maurel et al., 2014), autophagy may provide a more effective process than proteasomal degradation for removing supramolecular clusters of IRE1 $\alpha$ . Ubiquitination of IRE1 $\alpha$  has been associated with both IRE1 $\alpha$ 's activation (Zhu et al., 2014) as well as degradation (Gao et al., 2008; Sun et al., 2015), and it remains to be determined whether this optineurin-dependent autophagic process is dependent on ubiquitination (Wild et al., 2011; Khaminets et al., 2016). Recent studies have identified FAM134b as an ER-resident receptor that regulates ER membrane turnover by selective autophagy (Khaminets et al., 2015). Interestingly, silencing of *Fam134b* in MODE-K cells did not affect IRE1 $\alpha$  levels (not depicted), indicating that autophagy-dependent IRE1 $\alpha$  removal may be selective and regulated differently from the bulk process of ER-phagy.

The specific role for the unique expression of both IRE1 $\alpha$  and IRE1 $\beta$  in the intestinal epithelium has remained enigmatic. A clue comes from *Ern2*<sup>-/-</sup> mice, which are susceptible to DSS-induced colitis (Bertolotti et al., 2001). We now show that they complement each other's role in protein folding homeostasis within the intestinal epithelium. However, IRE1 $\alpha$  and IRE1 $\beta$  have divergent roles in organ inflammation. This feature is conspicuous in the *Atg16l1;Xbp1*-deficient epithelium and therefore likely reflects divergent effector functions that are independent of XBP1. These effectors appear particularly pertinent to the



**Figure 6. Selective autophagy receptors target IRE1 $\alpha$  for degradation.** (A) *Ern1* mRNA expression measured by qRT-PCR in ileal scrapings from the indicated genotypes and normalized to *Actb* expression.  $n = 15/11/10/11$ . The mean  $\pm$  SEM is shown. One-way ANOVA with Bonferroni's correction was used. (B) IRE1 $\alpha$  immunoblot (IB) of *Xbp1*-sufficient (MODE-K.*iCtrl*) and *Xbp1*-deficient (MODE-K.*iXbp1*) MODE-K cells co-cultured for 24 h with baflomycin (Baf), an inhibitor of vacuolar H $^{+}$  ATPase that prevents cargo degradation in autophagolysosomes. Data are representative of three independent experiments. (C) IRE1 $\alpha$  immunoblot after addition of protein synthesis inhibitor cycloheximide (CHX) and the autophagy inducer rapamycin (Rap) to MODE-K.*iCtrl* and MODE-K.*iXbp1* cells for 24 h. Data are representative of two independent experiments. (D–F) Coimmunoprecipitation (IP) of IRE1 $\alpha$  with selective

hyperactive IRE1 $\alpha$  observed in the *Atg16l1;Xbp1*-deficient epithelium (Maurel et al., 2014), and although their precise nature remains to be determined, it seems plausible that they might involve specific assembly of the UPR-osome, regulated IRE1-dependent decay, or cell death mechanisms (Hetz et al., 2011; Walter and Ron, 2011; Korennykh and Walter, 2012; Han et al., 2013).

In conclusion, the mechanism reported herein suggests biological integration of environmental and genetic risk across physiological processes that converge on pathological activation of IRE1 $\alpha$  as driver of ileal inflammation. Correspondingly, we show that *ATG16L1<sup>T300A</sup>*-related genetic risk associates with IRE1 $\alpha$  accumulation in ileal crypts. In this context, it is notable that NOD2, the singly strongest genetic risk factor of CD (Jostins et al., 2012) which, similar to *ATG16L1*, predisposes for CD involving the ileum (Cuthbert et al., 2002), links IRE1 $\alpha$  signaling with inflammation via a noncanonical pathway that does not involve the microbial NOD2 ligand muramyl dipeptide (Keestra-Gounder et al., 2016). It will be intriguing to explore whether IRE1 $\alpha$  signaling constitutes a common converging element across this and other CD-specific genetic risk factors that initiate the disease process in CD upon interaction with yet unknown environmental triggers.

## MATERIALS AND METHODS

### Mice

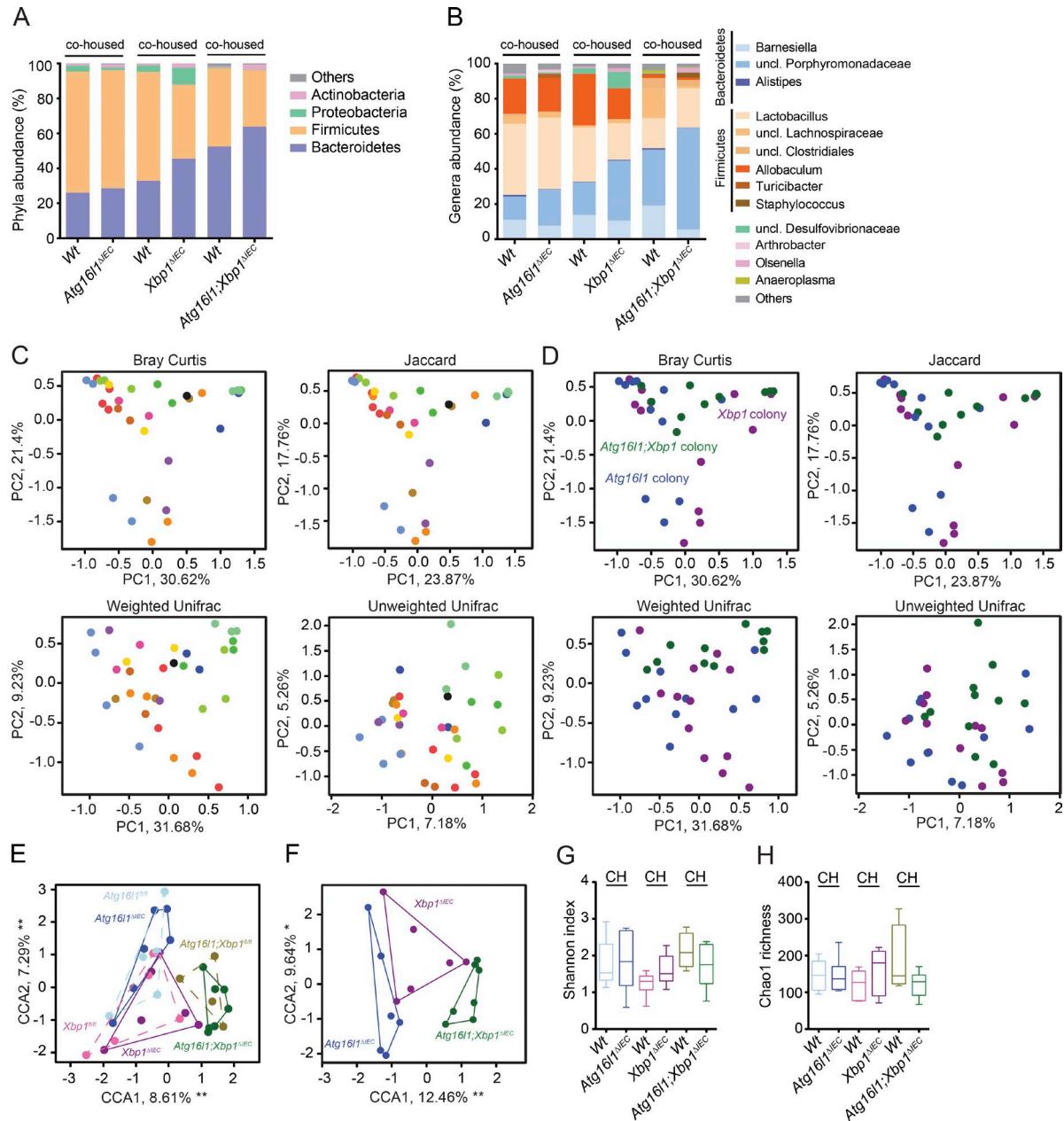
*Villin-Cre;Xbp1<sup>fl/fl</sup>* (*Xbp1<sup>ΔIEC</sup>*), *Villin-Cre;Atg16l1<sup>fl/fl</sup>* (*Atg16l1<sup>ΔIEC</sup>*), *Villin-Cre;Atg16l1<sup>fl/fl</sup>;Xbp1<sup>fl/fl</sup>* (*Atg16l1;Xbp1<sup>ΔIEC</sup>*), *Villin-Cre;Ern1<sup>fl/fl</sup>* (*Ern1<sup>ΔIEC</sup>*), *Ern2<sup>-/-</sup>*, and *Villin-Cre;Ern1<sup>fl/fl</sup>;Xbp1<sup>fl/fl</sup>* mice have been described previously (Bertolotti et al., 2001; Kaser et al., 2008; Adolph et al., 2013; Niederrreiter et al., 2013). *Villin-Cre;Atg16l1<sup>fl/fl</sup>;Ern1<sup>fl/fl</sup>* (*Atg16l1;Ern1<sup>ΔIEC</sup>*), *Villin-Cre;Xbp1<sup>fl/fl</sup>;Ern2<sup>-/-</sup>* (*Xbp1<sup>ΔIEC</sup>;Ern2<sup>-/-</sup>*), *Villin-Cre;Atg16l1<sup>fl/fl</sup>;Xbp1<sup>fl/fl</sup>;Ern1<sup>fl/fl</sup>* (*Atg16l1;Xbp1;Ern1<sup>ΔIEC</sup>*), *Villin-Cre;Atg16l1<sup>fl/fl</sup>;Xbp1<sup>fl/fl</sup>;Ern2<sup>-/-</sup>* (*Atg16l1;Xbp1<sup>ΔIEC</sup>;Ern2<sup>-/-</sup>*), *Villin-Cre;Ern1<sup>fl/fl</sup>;Ern2<sup>-/-</sup>* (*Ern1<sup>ΔIEC</sup>;Ern2<sup>-/-</sup>*), and *Villin-Cre;Ern1<sup>fl/fl</sup>;Xbp1<sup>fl/fl</sup>;Ern2<sup>-/-</sup>* (*Ern1<sup>ΔIEC</sup>;Xbp1<sup>ΔIEC</sup>;Ern2<sup>-/-</sup>*) mice were generated by intercrossing the relevant individual strains. All mice were on a C57BL/6 background except *Ern1<sup>fl/fl</sup>* (129/svJ × C57BL/6) and *Ern2<sup>-/-</sup>* (F1[DBA/2 × C57BL/6 × 129/svJ]) mice. The *Villin-Cre* transgene was maintained heterozygously. Mice were housed under SPF conditions at the central biomedical

services facility of the University of Cambridge if not stated otherwise. Whereas the *Atg16l1<sup>fl</sup>* allele in the aforementioned strains had exon 1 flanked by loxP sites (Adolph et al., 2013), a further strain (on C57BL/6 background) maintained under SPF conditions at the animal facility at Cedars-Sinai Medical Center (CSMC) had exon 3 floxed (*Atg16l1-ex3*; generated in collaboration with genOway) and was similarly crossed to *Villin-Cre* transgenic mice (on C57BL/6 background). Experiments were performed with age- and gender-matched mice and included littermate *Wt* animals as controls where technically possible. Cross-foster experiments were performed according to Fig. 8 (A, B, and G). For cross-foster experiments, breeding pairs from all genotypes were set up simultaneously, and pups of the indicated genotypes and littermate *Wt* controls were cross-fostered within 24 h upon birth and analyzed at 8–10 wk of age. Mice were handled and all experiments were performed according to institutional guidelines and the approval of relevant authorities (UK Home Office PPL80/2560, Austrian Federal Ministry of Science and Research, and CSMC Animal Care and Use Committee protocol 3723).

### Antibodies and reagents

The following antibodies were used for immunoblotting and immunoprecipitation: anti-IRE1 $\beta$  (Bertolotti et al., 2001), anti-phospho-IRE1 $\beta$  (gift from K. Kohno, Nara Institute of Science and Technology, Takayama, Ikoma, Nara, Japan), anti-IRE1 $\alpha$  (20790; H-190; polyclonal; Santa Cruz Biotechnology, Inc.), anti-IRE1 $\alpha$  (3294; 14C10), anti-NDP52 (9036; polyclonal), anti-NBR1 (9891; D2E6), anti-GAPDH (2118; 14C10), anti- $\beta$ -actin (4970; 13E5; Cell Signaling Technology), anti-optineurin (100000; polyclonal; Cayman), and anti-p62 (PM045; polyclonal; MBL). Immunofluorescence antibodies were anti-IRE1 $\alpha$  (20790; H-190), anti-lysozyme (27958; C19), anti-MUC2 (15334; H-300), anti-IRE1 $\beta$  (Bertolotti et al., 2001; Santa Cruz Biotechnology, Inc.), and anti-GRP78 (ab21685; polyclonal; Abcam). The following secondary antibodies were used: donkey anti-rabbit IgG (DyLight 488; ab96919; polyclonal), donkey anti-goat IgG (DyLight 488; ab96931; polyclonal), donkey anti-rabbit IgG (DyLight 650; ab96894; polyclonal; Abcam), and Clean-Blot (21230; Thermo Fisher Scientific). UEA-1 (RL-1062; Vector Laboratories) was used to stain secretory IECs.

autophagy receptors OPTN (D), NBR1 (neighbor of BRCA1 gene 1 protein; E), and p62 (F) in MODE-K.*iCtrl* and MODE-K.*iXbp1* cells with converse pull-downs also demonstrated.  $n = 2$ . Data are representative of three independent experiments. (G) Coimmunoprecipitation of IRE1 $\alpha$  with the selective autophagy receptor NDP52 in the human colorectal adenocarcinoma cell line HT29.  $n = 2$ . Data are representative of three independent experiments. (H) Coimmunoprecipitation and immunoblot of IRE1 $\alpha$  with the selective autophagy receptor NDP52 in HT29 cells at basal conditions (DMSO) and after pharmacological ER stress induction with tunicamycin (Tm).  $n = 3$ . Data are representative of two independent experiments. (I) IRE1 $\alpha$  and OPTN immunoblots after siRNA silencing of *Optn* in MODE-K.*iCtrl* and MODE-K.*iXbp1* cells.  $n = 2$ . Data are representative of two independent experiments. Ctrl, control. (J) IRE1 $\alpha$  immunoblot upon siRNA silencing of *Nbr1* or *Sqstm1* (p62) in MODE-K.*iXbp1* cells.  $n = 3$ . Data are representative of two independent experiments. (K) IRE1 $\alpha$  and NDP52 immunoblots upon siRNA silencing of *NDP52* in HT29 cells with and without tunicamycin treatment for 4 h.  $n = 2$ . Data are representative of two independent experiments. (L) Coimmunoprecipitation of OPTN with IRE1 $\alpha$  in ileal crypts of *Wt* and *Xbp1<sup>ΔIEC</sup>* mice.  $n = 3$ . Data are representative of two independent experiments.

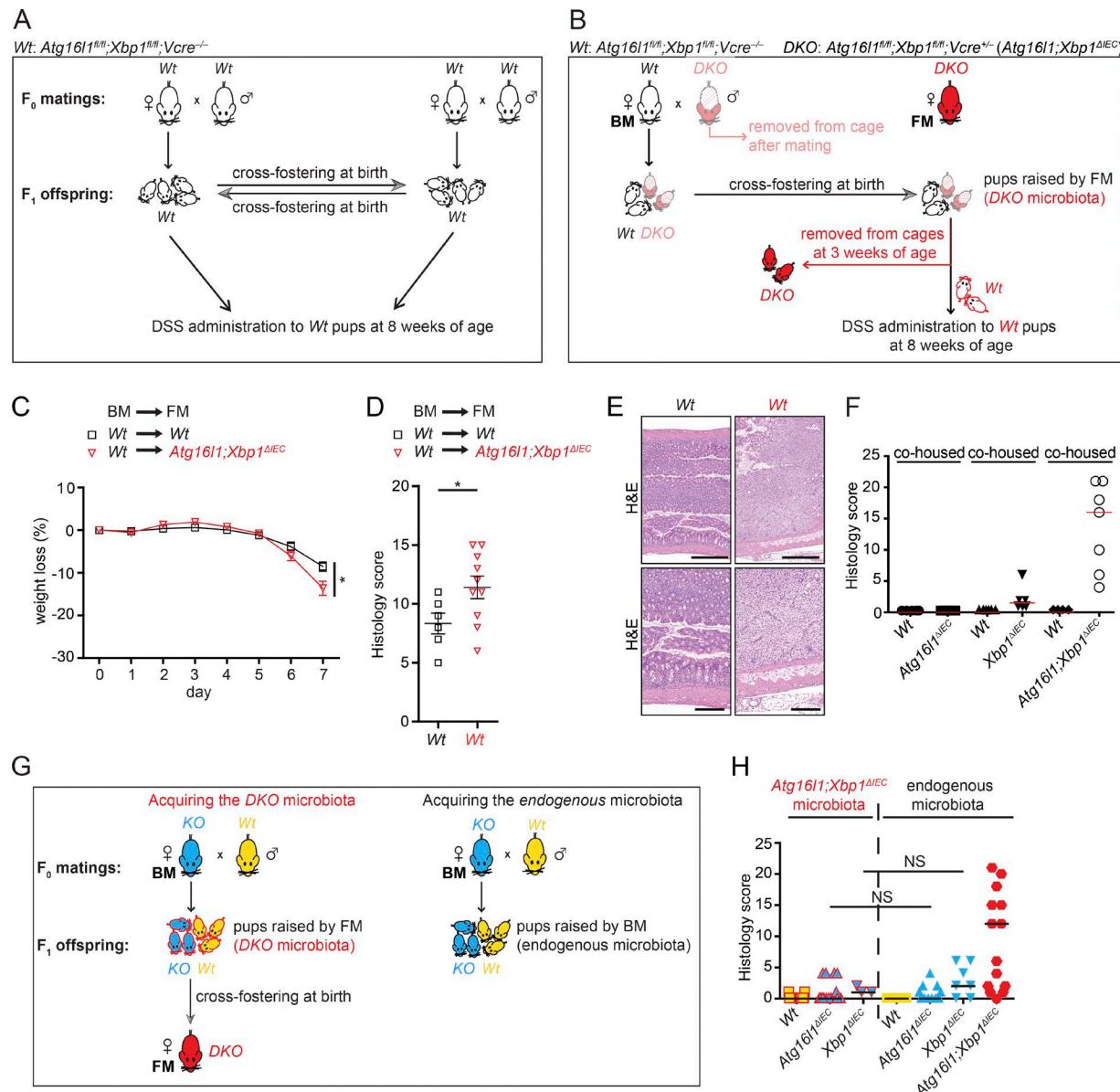


**Figure 7. *Atg16l1;Xbp1ΔIEC* mice harbor a dysbiotic and transmissible microbiota.** (A and B) Relative abundance of microbial taxa at phylum level ( $\geq 1\%$ ; A) and genus level ( $\geq 1\%$ ; B) representing ileal mucosa–adherent microbiota of 18-wk-old cohoused mutant (*V-cre<sup>+</sup>*) and *WT* littermates (*V-cre<sup>-</sup>*).  $n = 5/6/6/4/7$ . uncl., unclassified. (C and D) Ordination analysis of the constraining factors of the cage effect (each dot represents one mouse; cages are indicated as different colors; C) and genotype effect (each dot represents one mouse; colonies are indicated as different colors; D) in the microbiota analysis of cohoused mice based on principal coordinates analysis (Bray Curtis and Jaccard dissimilarity) and Unifrac (weighted and unweighted). *Atg16l1* colony,  $n = 11$ ; *Xbp1* colony,  $n = 12$ ; *Atg16l1;Xbp1* colony,  $n = 11$ . (E) CCA plot showing a significant genotype effect ( $F = 2.2270$ ;  $P = 0.001$ ) and gender effect ( $F = 2.2639$ ;  $P = 0.004$ ) in the indicated groups ( $n = 5/6/6/6/4/7$ ). Significant genotype effect is shown in D. Cage and litter effects were partialled out in this analysis and are shown in C. (F) CCA plot of knockouts only ( $n = 6/6/7$ ) constrained for mutation and gender effect (cage and litter effect were partialled out). (G and H)  $\alpha$  diversity estimates Shannon's index analyzing species evenness (G), and Chao 1 determining species richness (H) is shown ( $n = 5/6/6/4/7$ ). Statistical analysis was performed using nonparametric Kolmogorov-Smirnov test. CH, cohoused.

#### Analysis of microbial composition by 16S rDNA ribotyping

Bacterial DNA from ileal tissue–associated microbes was isolated using the Precellys 24 tissue homogenizer

(Bertin Technologies) and FastDNA SPIN kit for Soil (MP Biomedicals) according to the manufacturer's instructions. The V4 hypervariable region of the bacterial



**Figure 8. Microbiota of *Atg16l1;Xbp1<sup>ΔIEC</sup>* mice predisposes to colitis but not ileitis.** (A) Cross-fostering of Wt (*V-cre<sup>-/-</sup> Atg16l1<sup>fl/fl</sup>,Xbp1<sup>fl/fl</sup>*) offspring derived from a Wt (*V-cre<sup>-/-</sup> Atg16l1<sup>fl/fl</sup>,Xbp1<sup>fl/fl</sup>*) breeding pair to a Wt (*V-cre<sup>-/-</sup> Atg16l1<sup>fl/fl</sup>,Xbp1<sup>fl/fl</sup>*) foster dam and vice versa. Male breeders of the F<sub>0</sub> generation were removed from the cage before F<sub>1</sub> offspring were born. Cross-fostered offspring were subjected to 3% DSS-induced colitis for 7 d at 8 wk of age (this group of mice is displayed in black in panels C–E), performed in a mouse norovirus-free SPF animal facility (Cambridge). (B) Cross-fostering of Wt (*V-cre<sup>-/-</sup> Atg16l1<sup>fl/fl</sup>,Xbp1<sup>fl/fl</sup>*, displayed in black) and *Atg16l1;Xbp1<sup>ΔIEC</sup>* (DKO; *V-cre<sup>+</sup> Atg16l1<sup>fl/fl</sup>,Xbp1<sup>fl/fl</sup>*, displayed in shaded red) offspring derived from a hemizygous breeding pair (*V-cre<sup>-/-</sup> Atg16l1<sup>fl/fl</sup>,Xbp1<sup>fl/fl</sup>* [Wt] x *V-cre<sup>+</sup> Atg16l1<sup>fl/fl</sup>,Xbp1<sup>fl/fl</sup>* [DKO]) from a Wt (*V-cre<sup>-/-</sup> Atg16l1<sup>fl/fl</sup>,Xbp1<sup>fl/fl</sup>*) dam (biological mother; BM) to a *Atg16l1;Xbp1<sup>ΔIEC</sup>* (DKO; *V-cre<sup>+</sup> Atg16l1<sup>fl/fl</sup>,Xbp1<sup>fl/fl</sup>*) foster mother (FM; displayed in red). Male breeders of the F<sub>0</sub> generation were removed from the cage before F<sub>1</sub> offspring was born (displayed in shaded red). Before weaning of cross-fostered offspring, pups were genotyped, and *Atg16l1;Xbp1<sup>ΔIEC</sup>* (DKO) littermates were removed from the cage. Remaining Wt offspring that acquired *Atg16l1;Xbp1<sup>ΔIEC</sup>* microbiota (framed in red) were subjected to 3% DSS colitis for 7 d at 8 wk of age (this group of mice is displayed in red in panels C–E). (C) Relative weight loss after 3% DSS application for 7 d to 8-wk-old mice after cross-fostering Wt littermates either between Wt dams (displayed in black) or from a Wt dam to an *Atg16l1;Xbp1<sup>ΔIEC</sup>* dam after birth (displayed in red). Experimental design is shown in A and B. n = 6/10. The mean ± SEM is shown. Linear mixed-effects model fitted using R (lme package) was used. \*P < 0.05. (D and E) DSS colitis score (D) after 7 d of experiment performed in C with representative H&E images (E). n = 6/10. The mean ± SEM is shown. Unpaired two-tailed Student's t test was used. \*P < 0.05. Bars: (top) 500 μm; (bottom) 200 μm. (F) Histological score of cohoused mice of the indicated genotypes used for 16S rDNA ribotyping of ileal mucosa-adherent microbiota in Fig. 7 at 18 wk of age. n = 5/6/6/4/7. The median is shown. (G, left) Cross-fostering of either *Atg16l1<sup>ΔIEC</sup>* offspring (KO; displayed in blue) and their Wt littermates (displayed in yellow) or *Xbp1<sup>ΔIEC</sup>* offspring (KO; displayed in blue) and their Wt littermates (displayed in yellow) to an *Atg16l1;Xbp1<sup>ΔIEC</sup>* (DKO; displayed in red) foster dam allowing them to acquire the *Atg16l1;Xbp1<sup>ΔIEC</sup>* microbiota

16S rRNA gene was amplified as described previously (Kozich et al., 2013). The library was sequenced on an Illumina MiSeq system using the MiSeq Reagent kit (v3) with 2 × 250 bp Contig read length configuration and dual indexing. The data output was filtered and trimmed using Mothur package (Schloss et al., 2009), and chimeras were removed using the Uchime (Edgar et al., 2011) algorithm. The trimmed reads were aligned to the SILVA (Quast et al., 2013) reference database for excluding ambiguous sequences and were classified using the Riboosomal Database Project classifier (Wang et al., 2007). The aligned and subsampled datasets were used to compute a distance matrix for categorizing sequences into operational taxonomic units by a 97% similarity cut off. R software was used to analyze  $\alpha$  and  $\beta$  diversity (taxonomic classification and principal coordinates analysis plots). Clustering of 16S rDNA V4 ribotyping sequences was done at 3% genetic difference. Bray-Curtis and Jaccard dissimilarities were analyzed using constrained analysis of principle coordinates (capscale function in Vegan for plotting and ANOVA for significance test). The permutational (nonparametric) multivariate ANOVA (NPMANOVA; Adonis; Anderson, 2001; McArdle and Anderson, 2001) based on dissimilarities implemented in Vegan was used to test clustering of  $\beta$  diversity indices for ascertaining differences in community compositions. A p-value of significance was incorporated by comparing the obtained  $R^2$  values to that obtained from 1,000 random permutations of the data in each case. In addition, CCA was done to interpret the role of the variables and their multivariate associations among the datasets.

### Histology

Hematoxylin and eosin (H&E)-stained sections were assessed for intestinal inflammation using a semiquantitative composite scoring system previously described (Adolph et al., 2013). The total score represents the sum of five histological subscores multiplied by a factor that depends on the extent of the inflammation. The following histological subscores and grading were used (for each parameter: 0, absent; 1, mild; 2, moderate; 3, severe): mononuclear cell infiltrate (0–3), crypt hyperplasia (0–3), epithelial injury/erosion (0–3), polymorphonuclear cell infiltrates (0–3), and transmural inflammation (0, absent; 1, submucosal; 2, one focus extending into muscularis and serosa; 3, up to five foci extending into muscularis and serosa; 4, diffuse). The extent factor represents the proportion of intestine affected by intestinal inflammation: 1, <10%; 2, 10–25%; 3, 25–50%; and 4, >50%. Samples were evaluated in a blinded fashion.

(framed in red). (Right) Concurrently, non-cross-fostered *Atg16l1*<sup>1/IEC</sup> and *Xbp1*<sup>1/IEC</sup> offspring were raised by their respective biological dams, which allowed them to acquire endogenous microbiota. (H) Enteritis histology score of cross-fostered and *Atg16l1*; *Xbp1*<sup>1/IEC</sup> microbiota-acquired offspring compared with non-cross-fostered and endogenous microbiota acquired offspring at 8 wk of age.  $n = 21/18/3/12/13/7/16$ . The median is shown. Kruskal-Wallis with posthoc Holm's-corrected Mann-Whitney  $U$  test was used.

### DSS-induced colitis

At 8 wk of age, mice received 3% DSS (36–50 kD; MP Biomedicals) in their drinking water for 7 d. Mouse weight and rectal bleeding was assessed daily. Histological scoring was performed as described previously (Garrett et al., 2007).

### Crypt isolation

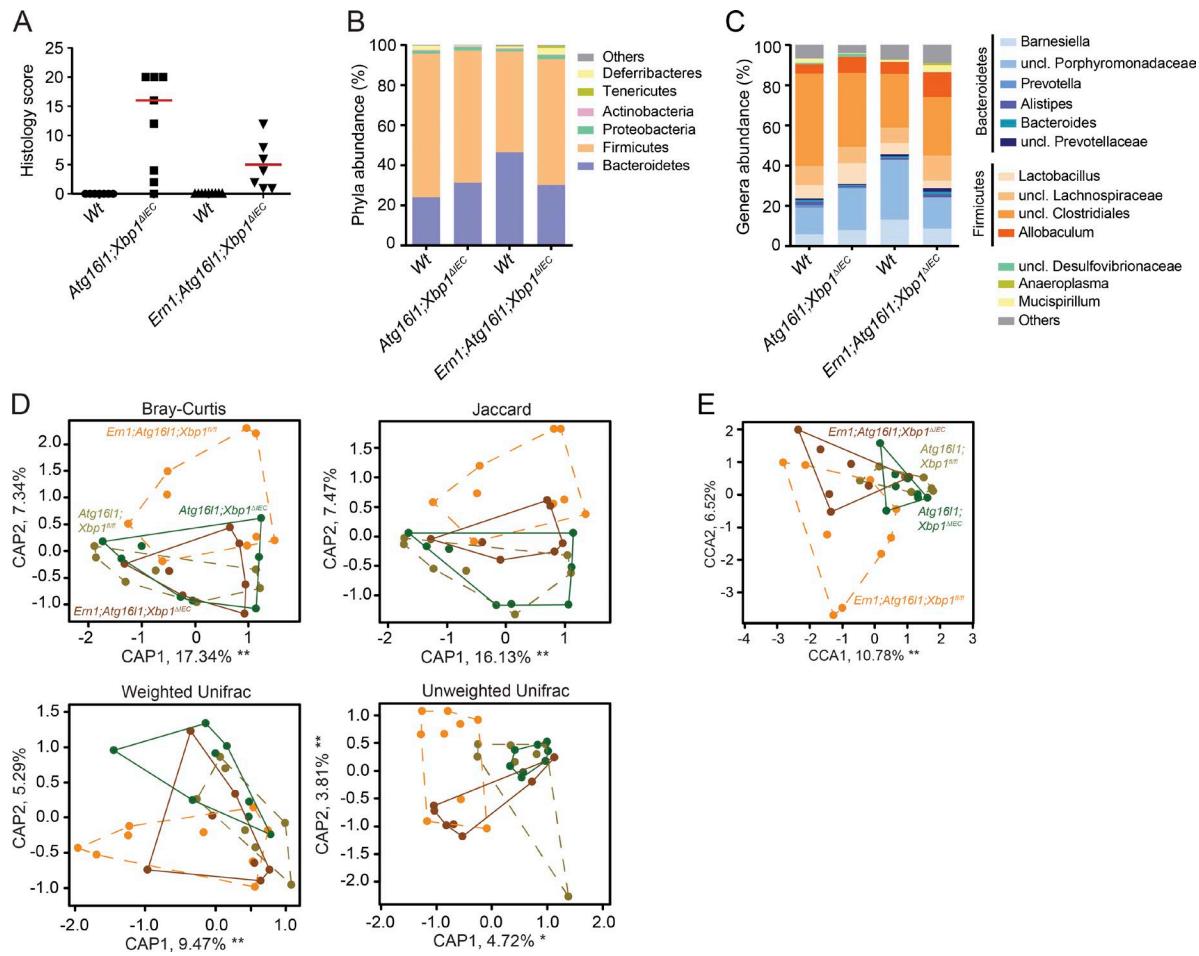
Crypt isolation protocol was adapted from Sato et al. (2009). In brief, flushed and longitudinally cut intestinal pieces were vortexed in ice-cold PBS for 5 min, transferred to 30 mM EDTA, and vortexed for 5 min and the supernatant was collected, which was repeated for a total of four times. Supernatants were microscopically checked for crypt enrichment and then spun down at 800 g and 4°C followed by RNA isolation (QIAGEN) or protein extraction.

### Immunohistochemistry, PAS, TUNEL, and BrdU staining

Immunohistochemistry on formalin-fixed paraffin-embedded sections was performed according to standard protocols as described previously (Adolph et al., 2013). In brief, sections were deparaffinized in Xylol and rehydrated in ethanol. Antigen retrieval was performed in citrate or EDTA buffer for 15 min at subboiling temperature, followed by quenching of endogenous peroxidase activity. Primary antibody was incubated at 4°C overnight, secondary biotinylated antibody for 1 h at room temperature, followed by streptavidin-HRP (Vector Laboratories). Sections were developed using a DAB Peroxidase Substrate kit (Vector Laboratories) and analyzed using a microscope (Axio Observer Z.1) with a camera (AxioCam MRC5) and AxioVision software (release 4.8; ZEISS) or AxioImager Z2 (AxioCam MRC5; ZEISS) with plan-Apochromat lenses and Zen Blue 2012 software. TUNEL using the TUNEL cell death detection kit (Roche) as per the manufacturer's instructions allowed us to quantify cell death by enumerating TUNEL<sup>+</sup> cells adjusted for the intestinal length of the respective sample. IEC proliferation was assessed 24 h after administering 1 mg BrdU (BD) intraperitoneally. Incorporated BrdU was detected by the BrdU in situ detection kit (BD), and BrdU<sup>+</sup> IECs were quantified along the crypt villus axis and presented as a ratio to total IECs along that axis. PAS staining was performed according to standard protocols.

### Immunofluorescence and confocal microscopy

Formalin-fixed paraffin-embedded sections were deparaffinized in Xylol and rehydrated in ethanol. Sections were antigen retrieved in citrate or EDTA buffer for 15 min at subboiling temperature, blocked (10% donkey serum; Bio-Rad Laboratories), and incubated with primary antibody for



**Figure 9. Similar microbiota composition despite amelioration of disease in *Ern1;Atg16l1;Xbp1<sup>Δ/ΔEC</sup>* mice.** (A) Histological score of mice used for 16S rDNA ribotyping of ileal mucosa–adherent microbiota in B–E at 10 wk of age.  $n = 7/8/9/7$ . The median is shown. (B and C) Relative abundance of microbial taxa at phylum level ( $\geq 10\%$ ; B) and genus level ( $\geq 10\%$ ; C) representing ileal mucosa–adherent microbiota of 10-wk-old *Ern1;Atg16l1;Xbp1<sup>Δ/ΔEC</sup>* mice and their *Wt* littermates compared with *Atg16l1;Xbp1<sup>Δ/ΔEC</sup>* mice.  $n = 7/8/9/7$ . uncl., unclassified. (D) Ordination analysis including all constraining factors (genotype, mutation, gender, litter, and cage) based on Bray–Curtis dissimilarity (genotype:  $R^2 = 0.16566$ ,  $P = 0.1219$ ; gender:  $R^2 = 0.01154$ ,  $P = 0.9121$ ; litter:  $R^2 = 0.08843$ ,  $P = 0.8472$ ; and cage:  $R^2 = 0.17390$ ,  $P = 0.4535$ ), Jaccard index (genotype:  $R^2 = 0.14477$ ,  $P = 0.1129$ ; gender:  $R^2 = 0.01465$ ,  $P = 0.9700$ ; litter:  $R^2 = 0.10618$ ,  $P = 0.8252$ ; and cage:  $R^2 = 0.16193$ ,  $P = 0.5614$ ), weighted Unifrac distances (genotype:  $F = 1.0380$ ,  $P = 0.372$ ; gender:  $F = 1.1375$ ,  $P = 0.247$ ; litter:  $F = 0.9034$ ,  $P = 0.634$ ; and cage:  $F = 1.0281$ ,  $P = 0.411$ ) and unweighted Unifrac distances (genotype:  $F = 1.0604$ ,  $P = 0.172$ ; gender:  $F = 1.0816$ ,  $P = 0.217$ ; litter:  $F = 1.0265$ ,  $P = 0.276$ ; and cage:  $F = 1.0270$ ,  $P = 0.304$ ).  $n = 7/8/9/7$ . (E) Constrained CCA plot partialled out for cage and litter effect showed a marginal significance for genotype ( $F = 1.7166$  and  $P = 0.045$ ).  $n = 7/8/9/7$ .

1 h at room temperature. Fluorescently labeled secondary antibodies were incubated for 45 min at room temperature, and slides were mounted with Prolong Gold Antifade reagent with DAPI (Invitrogen). Images were acquired using a confocal microscope (SP5; Leica Biosystems) and LAS AF software.

#### Transmission electron microscopy

Intestines were harvested and flushed with saline, cut into thin sections, and fixed in 4% glutaraldehyde in 0.1 M Hepes buffer, pH 7.4, for 12 h at 4°C. Sections were rinsed in 0.1 M Hepes buffer for five times and treated with 1% osmium ferricyanide at room temperature for 2 h, followed by rinsing in deionized water for five times and treated with 2% uranyl acetate in

0.05 M maleate buffer, pH 5.5, for 2 h at room temperature. Samples were rinsed in deionized water and dehydrated in 70–100% ethanol, followed by treatment with two changes of dry acetonitrile and infiltration with Quetol epoxy resin. Images were taken with a transmission electron microscope (Tecnai G2; FEI) operated at 120 Kv equipped with a digital camera (AMT XR60B) running Deben software.

#### Human intestinal biopsies

After obtaining written informed consent, human intestinal biopsies from the terminal ileum of 31 CD patients and 43 healthy controls were collected upon colonoscopy and stored in optimal cutting temperature compound. DNA

from EDTA blood samples was extracted (Wizard Genomic DNA purification kit; Promega) according to the manufacturer's instructions, and genotyping for *ATG16L1* (T300A) SNP (rs2241880) was performed as described previously (Deuring et al., 2014). In brief, a 330-bp fragment containing the *ATG16L1* (T300A) SNP was amplified using Phusion High-Fidelity PCR (Phusion; Thermo Fisher Scientific) and the following primers: forward, 5'-TTGGAGTCCACA GGTTAG-3'; and reverse, 5'-CAGTAGCTGGTACCCCTCA CTTC-3' (Deuring et al., 2014). Subsequently, the DNA fragment was gel extracted from a 1% agarose gel using a Gel Extraction kit (QIAGEN). The forward primer was used for sequencing of the 330-bp *ATG16L1* (T300A) amplicon at Source Bioscience Cambridge. All protocols were approved by Cambridgeshire 4 Research Ethics Committee (reference 03/5/012). Patient characteristics are outlined in Table S1.

#### Cell culture

MODE-K cells cultured in DMEM were transduced with an *Xbp1*-specific or control shRNA lentiviral vector as described previously (Kaser et al., 2008), and stable clones were established. HT29 cells were cultured in DMEM supplemented with 10% FBS and 5% PenStrep (Gibco). Cells were harvested after seeding in 6-well plates overnight or 72 h after transfection with *Nbr1*, *NDP52*, *Optn*, *Sqstm1*, or control siRNA (Thermo Fisher Scientific). 50 nM bafilomycin (Sigma-Aldrich), 10 µg/ml cycloheximide (Sigma-Aldrich), 2 µg/ml tunicamycin (Sigma-Aldrich), or 50 nM rapamycin (Sigma-Aldrich) was added to the cell culture for the indicated time.

#### Silencing via siRNA

siRNA experiments of *Nbr1*, *NDP52*, *Optn*, *Sqstm1*, or control siRNA (Thermo Fisher Scientific) were conducted according to the manufacturer's instructions. In brief, MODE-K and HT29 cells were cultured to ~60% confluence in 6-well plates and transfected with siRNAs suspended in 100 µl Opti-MEM (Gibco) and 6 µl Lipofectamine 2000 (Thermo Fisher Scientific). After 72 h, cultures were lysed in radioimmunoprecipitation assay (RIPA) buffer or Triton X buffer and analyzed by immunoblotting.

#### RNA extraction and quantitative RT-PCR (qRT-PCR)

RNA was isolated from ileal scrapings or crypt fractions using an RNeasy Mini kit (QIAGEN) and reverse transcribed to cDNA using M-MLV reverse transcriptase (Invitrogen). qRT-PCR was performed with SYBR green mastermix (Eurogentec) on an MX-3005 system (Agilent Technologies). For qRT-PCR, the following pairs of primers were used: *mActb* (α-actin), 5'-GATGCTCCCCGGGCTGTATT-3' and 5'-GGGGTACTTCAGGGTCAGGA-3' (Kaser et al., 2008); *mErn1*, 5'-GCTCCAGTTTCCAGGATG-3' and 5'-GAGCCACCTTGTAGGTTCTG-3' (designed in this study); *mErn2*, 5'-GATGAGGCAGCAGCAGC-3' and 5'-GCCCTGCTTCAGGGTCAT-3' (designed in this study);

and *mHprt*, 5'-GTTAAGCAGTACAGCCCCAAA-3' and 5'-AGGGCATATCCAACAACAAACTT-3' (Amthor et al., 2009). Target gene expression was normalized to house-keeping gene expression.

#### Xbp1 splicing assay

Assessment of *Xbp1* splicing status by PCR was described previously (Kaser et al., 2008). In brief, isolated RNA was reverse transcribed (M-MLV reverse transcriptase; Invitrogen), amplified by PCR (HotStarTaq; QIAGEN) using the primers *mXbp1* sp. forward 5'-ACACGCTTGGGAATGGACAC-3' and *mXbp1* sp. reverse 5'-CCATGGGAAGATGTTCTGG-3' (Iwakoshi et al., 2003), resolved on a 2.5% agarose gel, and spliced *Xbp1* (171 bp) and unspliced *Xbp1* (145 bp) products were assessed.

#### Immunoblot analysis

Immunoblot analysis was performed according to standard procedures. In brief, cells, IEC scrapings, or isolated crypts were lysed using RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) or Triton X buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 0.1% SDS, and 0.5% deoxycholate), supplemented with protease and phosphatase inhibitors (Thermo Fisher Scientific). Equal protein amounts eluted in Laemmli buffer were denatured at 95°C and resolved on 8–12.5% SDS-PAGE (Bio-Rad Laboratories), transferred to polyvinylidene fluoride membrane (Bio-Rad Laboratories), blocked with 5% skim milk or 5% BSA in TBS-Tween, and incubated with primary antibody in 5% BSA TBS-Tween overnight. Antibody was detected with HRP-conjugated secondary antibody and visualized with LumiGLO (Cell Signaling Technology).

#### Immunoprecipitation

Protein A sepharose beads (GE Healthcare) were washed in PBS and in RIPA lysis buffer or Triton X buffer at 4°C overnight. 60 µl of washed sepharose beads resuspended in RIPA or Triton X buffer containing protease and phosphatase inhibitors (Thermo Fisher Scientific) was added to the protein lysates and incubated for 30 min at 4°C. Subsequently, the precleared lysates were incubated with pull-down antibody or with IgG control antibody for 1 h at 4°C, before 60 µl of washed protein A sepharose beads was added and incubated for 1 h at 4°C. After washing the samples in RIPA or Triton X buffer containing protease and phosphatase inhibitors, samples were resuspended in 70 µl of 2× loading buffer before they were boiled at 95°C for 5 min, incubated on ice for 5 min, and run on 10% SDS PAGE.

#### Statistical analysis

Statistical significance was tested by an unpaired two-tailed Student's *t* test or a Mann-Whitney *U* test as appropriate and considered significant at *P* < 0.05. In experiments where more than two groups were compared, Kruskal-Wallis fol-

lowed by Mann-Whitney *U* and posthoc Bonferroni Holm's correction or one-way ANOVA with posthoc Bonferroni correction were performed as appropriate. Prism (version 6.0d; GraphPad Software) was used for data analysis.

## Online supplemental material

Table S1 is available as an Excel file and shows the characteristics of all patients analyzed for IRE1 $\alpha$  immunoreactivity, stratified by ATG16L1 genotype.

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Author contributions: M. Tschurtschenthaler, T.E. Adolph, L. Niederreiter, J.W. Ashcroft, P. Rosenstiel, R.S. Blumberg, and A. Kaser conceived and designed the experiments. M. Tschurtschenthaler, J.W. Ashcroft, T.E. Adolph, and L. Niederreiter together with S. Saveljeva, J. Bhattacharyya, M.B. Flak, and G.M. Fuhler performed and analyzed most experiments. R. Bharti and P. Rosenstiel performed and analyzed 16S rRNA sequencing experiments. M. Parkes supervised collection of human biopsies. K. Kohno, T. Iwawaki, A.M. Smith, H.P. Harding, and D. Ron provided essential reagents and mouse strains. D.Q. Shih and S.R. Targan generated and analyzed new mutant mouse strains. G.M. Fuhler, C.J. van der Woude, and M.P. Peppelenbosch contributed analytic methods. A. Kaser devised and coordinated the project and together with M. Tschurtschenthaler, T.E. Adolph, L. Niederreiter, P. Rosenstiel, and R.S. Blumberg with contributions from all authors, wrote the manuscript.

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