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Defective ATG16L1-mediated removal of IRE1α drives Crohn’s disease–like ileitis

Markus Tschurtschenthaler, Timon E. Adolph, Jonathan W. Ashcroft, Lukas Niederreiter, Richa Bharti, Svetlana Saveljeva, Joya Bhattacharyya, Magdalena B. Flak, David Q. Shih, Gweny M. Fuhler, Miles Parkes, Kenji Kohno, Takao Iwawaki, C. Janneke van der Woude, Heather P. Harding, Andrew M. Smith, Maikel P. Peppelenbosch, Stephan R. Targan, David Ron, Philip Rosenstiel, Richard S. Blumberg, and Arthur Kaser

ATG16L1[Superscript 7300A], 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 IRE1α. IRE1α accumulates in Paneth cells of Atg16l1[Δ7300C] mice, and humans homozygous for ATG16L1[Superscript 7300A] exhibit a corresponding increase of IRE1α in intestinal epithelial crypts. In contrast to a protective role of the IRE1β isoform, hyperactivated IRE1α also drives a similar ileitis developing earlier in life in Atg16l1;Xbp1[Δ401C] mice, in which ER stress is induced by deletion of the unfolded protein response transcription factor XBP1. The selective autophagy receptor optineurin interacts with IRE1α, and optineurin deficiency amplifies IRE1α levels during ER stress. Furthermore, although dysbiosis of the ileal microbiota is present in Atg16l1;Xbp1[Δ401C] 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 IRE1α 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, following 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[Superscript 7300A] is particularly noteworthy (Hampe et al., 2007; Rioux et al., 2007) as it has led to the...
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). However, increasing and prolonged ER stress causes IRE1α activation to transition from an adaptive to a maladaptive, pro-death and proinflammatory UPR, associated with IRE1α forming homodimers and large clusters, respectively (Li et al., 2010; Korennykh and Walter, 2012; Ghosh et al., 2014). Whereas IRE1α was barely detectable in Vil-Cre<sup>Cre<sup>Wt</sup> littermate controls, IEC-specific deletion of Atg16l1 or Xbp1 in 10-wk-old Atg16l1<sup>IEC</sup> and Xbp1<sup>IEC</sup> mice, respectively, led to increased IRE1α expression and phosphorylation compared with lysates from Xbp1<sup>IEC</sup> mice (Adolph et al., 2013), but it has remained unclear whether this has a role in transmural ileitis. IRE1α is the ER transmembrane receptor that responds to protein misfolding by activating the XBP1 branch of the UPR, aiming 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 IRE1α activation to transition from an adaptive to a maladaptive, pro-death and proinflammatory UPR, associated with IRE1α forming homodimers and large clusters, respectively (Li et al., 2010; Korennykh and Walter, 2012; Ghosh et al., 2014). Whereas IRE1α was barely detectable in Vil-Cre<sup>Cre<sup>Wt</sup> littermate controls, IEC-specific deletion of Atg16l1 or Xbp1 in 10-wk-old Atg16l1<sup>IEC</sup> and Xbp1<sup>IEC</sup> mice, respectively, led to increased IRE1α immunoreactivity specifically in small intestinal crypts, with the highest levels detected when both Atg16l1 and Xbp1 were absent as observed in Atg16l1<sup>IEC</sup> and Xbp1<sup>IEC</sup> mice (Fig. 2 A). Furthermore, IRE1α immunofluorescence localized most predominantly to Paneth cells, which were identified by antilysozyme and by lectin Ulex europaeus agglutinin 1 (UEA-1) costaining (Fig. 2, B
and C). Other highly secretory IECs such as goblet cells, where protein misfolding might be prevalent, did not exhibit increased IRE1α immunoreactivity upon Atg16l1 and/or Xbp1 deletion (Fig. 2, A–C). Whereas IRE1α (encoded by Ern1) at low levels is ubiquitously expressed, the intestinal and bronchial epithelium expresses a paralogue with high sequence similarity, IRE1β (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 IRE1β mostly in crypt but also in villus IECs of Xbp1ΔIEC and Atg16l1;Xbp1ΔIEC but not in Atg16l1ΔIEC 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 IRE1α and IRE1β to CD-like ileitis in Atg16l1;Xbp1ΔIEC mice, we generated Ern1−/−;Atg16l1;Xbp1ΔIEC and Ern2−/−;Atg16l1;Xbp1ΔIEC mice, respectively. Fig. 2 (F and G) demonstrates that ileitis is prevented by IEC-specific deletion of Ern1, whereas germine 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;Xbp1ΔIEC parent strain has been rederived (Fig. 2 H). Protection from ileitis afforded by IEC-specific elimination of IRE1α function in Ern1−/−;Atg16l1;Xbp1ΔIEC mice was also associated with a decrease in the number of dying TdT-mediated dUTP nick-end labeling (TUNEL)+ IECs (Fig. 2, I and J) but, importantly, not with rescue of impaired lysozyme staining in Paneth cells compared with Atg16l1;Xbp1ΔIEC 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 IRE1α as driver of CD-like ileitis and IRE1β providing a protective function.

**Figure 1.** 35-wk-old Atg16l1ΔIEC mice exhibit increased ER stress and transmural CD-like ileitis. [A] Representative images of GRP78 (green) immunoreactivity in 10- and 35-wk-old Atg16l1ΔIEC mice. n = 3. DAPI is shown in blue. Bars, 50 µm. [B and C] Representative H&E images (B) and enteritis histology score of 35-wk-old Atg16l1ΔIEC 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 Atg16l1ΔIEC compared with Wt mice. Bars, 100 µm. [D] Representative H&E images of 9–10-mo-old Atg16l1−/−Xbp1ΔIEC 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−/−Xbp1ΔIEC mice. n = 10. Bars, 100 µm.

**IRE1α drives CD-like ileitis in 35-wk-old Atg16l1ΔIEC mice**

Absence of XBP1 function abolishes cells’ capacity for effective proteostasis causing ER stress, as observed in Xbp1ΔIEC mice, in turn triggering profound IRE1α activation (Kaser et al., 2008). This accounts for the mild, superficial enteritis that develops in Xbp1ΔIEC mice (Adolph et al., 2013). The observation that IRE1α accumulates most profoundly in Atg16l1;Xbp1ΔIEC Paneth cells and drives the transmural CD-like ileitis in Atg16l1;Xbp1ΔIEC mice suggested that autophagy may be directly involved in regulating IRE1α function.
Figure 2. **IRE1α controls CD-like inflammation.** (A) Representative images of IRE1α immunohistochemistry (brown) on ileal sections of 10-wk-old *Atg16l1*^ΔIEC^, *Xbp1*^ΔIEC^, and *Atg16l1*;*Xbp1*^ΔIEC^ mice. *n* = 3. Hematoxylin is in blue. Bars, 20 µm. (B) Representative confocal images of IRE1α immunofluorescence (green) and Paneth cell–derived lysozyme (red) on ileal sections from the indicated genotypes at 10 wk of age. Note the perinuclear cytoplasmic IRE1α immunoreactivity of Paneth cells from *Atg16l1*^ΔIEC^ mice that are lysozyme positive on the luminally oriented apex. *n* = 3. DAPI is in blue. Bars, 20 µm.
However, it remained possible that decreased autophagy function and overactivation of IRE1α 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 Atg16l1ΔIEC mice (Fig. 1, B and C) provided us an opportunity to test the hypothesis that IRE1α activity is directly dependent on autophagy function. IRE1α immunofluorescence at the small intestinal crypt bottom was indeed observed to increase from age 10–35 wk in Atg16l1ΔIEC mice, which contrasts with equally minimal staining in Wt littermates of either age (Fig. 3 A). Therefore, we generated Ern1;Atg16l1ΔIEC mice and studied them at 35 wk of age. In marked contrast to Atg16l1ΔIEC mice of the same age, 35-wk-old Ern1;Atg16l1ΔIEC 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 IRE1α activity and that IRE1α 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 IRE1α-dependent spontaneous ileitis.

IRE1α accumulates in crypts of individuals homozygous for ATG16L1T300A

The ATG16L1T300A 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 (Pescott et al., 2007). Based on the identification of IRE1α as driver of CD-like ileitis in 35-wk-old Atg16l1ΔIEC mice, we assessed IRE1α expression in nonflamed 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 ± 7.6% of crypts positive for IRE1α staining in individuals with G/G, compared with 19.4 ± 3.7% and 23.19 ± 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 ATG16L1T300A may trigger an exaggerated IRE1α response.

Altogether, these data establish a critical role of IRE1α 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 IRE1α and IRE1β relate to each other within the intestinal epithelium and contribute to mucosal homeostasis? Second, how does IRE1α 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?

IRE1α and IRE1β are cross-compensatory in the intestinal epithelium

IRE1α and IRE1β are ER transmembrane receptors, which form homodimers in the presence of misfolded proteins, triggering their kinase and endoribonuclease function (Walter and Ron, 2011). IRE1α’s endoribonuclease primarily splices Xbp1 mRNA, causing a frame shift that converts XBP1 into its endoribonuclease primary splices XBP1 into the active transcription factor, whereas IRE1β predominantly engages in regulated IRE1-dependent decay that degrades specific (m)RNAs on the ER membrane (Hetz et al., 2011). Active IRE1α (and presumably IRE1β) forms a scaffold and recruits adapters and signaling molecules (Woehlbi and Hetz, 2011), and upon unabated, unresolvable stress, IRE1α 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 IRE1α and IRE1β 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 IRE1α at the crypt base in Ern2−/− and conversely for IRE1β in Ern1ΔIEC 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 Ern1ΔIEC and Ern1ΔIEC,Ern2−/− lysates (Fig. 4 E). Notably, Xbp1 splicing was markedly increased in Ern2−/− ileal crypt lysates (Fig. 4 E), indicating IRE1α overactivation in the absence of IRE1β function. Deletion of the floxed exon 2 of Xbp1 results in IRE1α overactivation as observed through IRE1α accumulation and phosphorylation.

(C) Representative confocal images of IRE1α immunofluorescence (green) and the Paneth and goblet cell–type–specific lectin UA-1 (red) on ileal sections from indicated genotypes at 10 wk of age. n = 3. DAPI is in blue. Bars, 20 µm. (D) Representative confocal images of IRE1β immunofluorescence (green) of the indicated genotypes at 10 wk of age. Note the specific IRE1β immunoreactivity of crypts (insets, bottom) and villus IECs from Atg16l1loxP/loxP and Xbp1ΔIEC mice. DAPI is in blue. n = 3. Bars: (top) 50 µm; (bottom) 20 µm. (E) IRE1β immunoprecipitation (IP) and immunoblot (IB) as well as pIRE1β 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 µm. (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 ± SEM is shown. Mann–Whitney U test was used. DAPI is in blue. Bars, 20 µm. *, P < 0.05; **, P < 0.01; ****, P < 0.0001.
Hypomorphic ATG16L1 triggers IRE1α-mediated ileitis | Tschurtschenthaler et al.

Massive splicing of Xbp1 mRNA (the splicing site encoded by exon 4 is intact in the Xbp1ΔIEC allele; Fig. 4E), as previously reported (Kaser et al., 2008; Niederreiter et al., 2013). Ern1;Xbp1ΔIEC ileal crypts exhibited some residual Xbp1 splicing, which was completely abrogated in Ern1;Atg16l1ΔIEC;Ern2−/− ileal crypts (Fig. 4E), indicating that IRE1β may contribute to Xbp1 splicing. Consistent with a cross-compensatory role of IRE1α and IRE1β, Ern1ΔIEC;Ern2−/− 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 Xbp1ΔIEC mice (Kaser et al., 2008), whereas single-mutant Ern1ΔIEC and Ern2−/− crypts were almost indistinguishable from Wt controls with only minimal distension of the ER (Fig. 4, F and G). Ern1ΔIEC;Ern2−/− mice exhibited an absence of lysozyme immunofluorescence reflecting the massive alterations in the secretory compartment observed in comparison with Ern1ΔIEC and Ern2−/− 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 Ern1ΔIEC;Ern2−/− mice, with decreased MUC2 staining observed in Ern2−/− single-mutant mice (Fig. 5, C and D), consistent with an important role of IRE1β in mucin secretion (Tsuru et al., 2013). Whereas Ern1ΔIEC;Ern2−/− 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 Ern1ΔIEC and Ern2−/− single-mutant mice (Fig. 5, E–J). Together, these data demonstrate that IRE1α and IRE1β
complement each other to maintain Paneth cell and intestinal homeostasis, although their roles fundamentally diverge in Atg16l1;Xbp1ΔIEC mice, with IRE1α driving and IRE1β protecting from CD-like ileitis.

**IRE1α accumulates in the absence of selective autophagy receptor optineurin**

The observed increased IRE1α activity in Atg16l1ΔIEC, Xbp1ΔIEC, and Atg16l1;Xbp1ΔIEC mice could not be explained by transcriptional regulation of Ern1 (Fig. 6 A), suggesting that autophagy may be involved in IRE1α protein turnover. Autophagy engulfs intracellular material, e.g., organelles (mitophagy, pexophagy, and ER-phagy), macromolecular complexes (aggregophagy), 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 IRE1α 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. Bafilomycin A, an inhibitor of vacuolar H+ ATPase that prevents acidification of autophagolysosomes and hence degradation of their cargo, increased IRE1α 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 IRE1α protein levels in MODE-K.iXbp1 but not MODE-K.iCtrl cells, whereas inhibition of protein translation via cycloheximide decreased IRE1α levels in both cell lines (Fig. 6 C). This suggested that autophagy may contribute to IRE1α turnover predominantly during ER stress.

Considering the formation of large clusters of IRE1α during profound and sustained ER stress, we hypothesized that IRE1α 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., 2015). As reductionist model systems to study the involvement of autophagy in IRE1α 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. Bafilomycin A, an inhibitor of vacuolar H+ ATPase that prevents acidification of autophagolysosomes and hence degradation of their cargo, increased IRE1α 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 IRE1α protein levels in MODE-K.iXbp1 but not MODE-K.iCtrl cells, whereas inhibition of protein translation via cycloheximide decreased IRE1α levels in both cell lines (Fig. 6 C). This suggested that autophagy may contribute to IRE1α turnover predominantly during ER stress.

Mice with CD-like ileitis harbor a dysbiotic microbiota

The spontaneous CD-like ileitis observed in Atg16l1;Xbp1ΔIEC 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;Xbp1ΔIEC) co-housed with their Vil-CreEG Fl+ Wt [Atg16l1ΔIEC;Xbp1ΔIEC littermates] exhibited an overall distinct tissue-adherent ileal microbial composition compared with Atg16l1ΔIEC and their Wt [Atg16l1ΔIEC] littermates) or Xbp1ΔIEC and Wt [Xbp1ΔIEC] littermates) colonies (adonis; Bray-Curtis: genotypes, R 2 = 0.14812, P = 0.000999; gender, R 2 = 0.07445, P = 0.004995; litter, R 2 = 0.064, P = 0.99; Fig. 7, A–F), with the maximum microbial shift being observed between Atg16l1;Xbp1ΔIEC and Atg16l1ΔIEC mice (adonis; Bray-Curtis: R 2 = 0.22912, P = 0.01598; Jaccard: R 2 = 0.18951, P = 0.01199). Specifically, the Atg16l1;Xbp1ΔIEC 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 Atg16l1ΔIEC mice (Fig. 7, A and B). Differences in bacterial composition were further highlighted by a relatively small overlap of operational taxonomic units between Atg16l1ΔIEC, Xbp1ΔIEC, and Atg16l1;Xbp1ΔIEC mice, respectively (not depicted), whereas species diversity and species richness were comparable between respective colonies.
Figure 4. Cross-compensatory up-regulation of IRE1α and IRE1β maintains Paneth cell homeostasis. (A and B) Representative confocal images of IRE1α (A) or IRE1β (B) immunofluorescence (green) of the indicated genotypes at 10 wk of age. Note the cross-compensatory up-regulation of IRE1β and IRE1α in crypts of Ern1ΔIEC and Ern2−/− mice, respectively (white arrows). n = 3. DAPI is in blue. Bars, 20 µm. (C and D) Ern1 (C) and Ern2 (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 ± SEM is shown. One-way ANOVA with Bonferroni’s correction was used. Note that IRE1α and IRE1β (encoded by Ern1 and Ern2) in Ern2−/− and Ern1ΔIEC mice, respectively, are not transcriptionally regulated. (E) Xbp1 splicing in crypt IECs from the indicated genotypes indicative of upstream activation of IRE1α/IRE1β. Note increased
Acquisition of an altered microbiota by Wt mice co-housed with their IEC-mutant littermates afforded an opportunity to dissect functional consequences via cross-fostering studies. Wt litters cross-fostered by Atg16l1ΔIEC mice indeed exhibited increased severity of colitis induced by dextran sodium sulfate (DSS), compared with Wt mice cross-fostered with Wt litters (Fig. 8, A–E). Importantly, all Wt litters and Wt foster dams were V-Cre<sup> Cre<sup>−/−</sup></sup>: Atg16l1ΔIEC mice, hence stemming from the Atg16l1ΔIEC colony. Altogether, these data established that the Atg16l1ΔIEC microbiota is indeed dysbiotic and aggravates exogenously induced experimental colitis.

Ileal dysbiosis does not trigger ileitis

Wt mice co-housed with their Atg16l1ΔIEC litters did not develop any intestinal inflammation, nor did co-housed Wt litters of Xbp1ΔIEC mice (Fig. 8 F), despite an almost identical ileal microbial ecology between Wt mice and their respective IEC-mutant litters (Fig. 7, A and B). Having established the dysbiotic, aggravating effect of the Atg16l1ΔIEC; Xbp1ΔIEC microbiota on DSS colitis (Fig. 8, C–E) and the transferability of the microbiota between IEC-mutant mice, their Wt litters, 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 Atg16l1ΔIEC or Xbp1ΔIEC mice. Cross-fostering of Wt, Atg16l1ΔIEC, and Xbp1ΔIEC litters with Atg16l1ΔIEC; Xbp1ΔIEC 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 Atg16l1ΔIEC; Xbp1ΔIEC litters cross-fostered with Atg16l1ΔIEC; Xbp1ΔIEC foster dams did not develop CD-like ileitis as observed in Atg16l1ΔIEC; Xbp1ΔIEC mice or 35-wk-old Atg16l1ΔIEC mice. Consistent with ileal dysbiosis not triggering ileal inflammation in this model, Ern1<sup>−/−</sup>; Atg16l1ΔIEC; Xbp1ΔIEC mice, which were protected from ileitis (Fig. 2, F and G; and Fig. 9 A), exhibited an ileal microbiota similar to Atg16l1ΔIEC; Xbp1ΔIEC mice (Fig. 9, B–E) and Paneth cells that remained profoundly impaired in lysozyme staining (Fig. 2, K and L). Specifically, β diversity did not show any differences between Ern1<sup>−/−</sup>; Atg16l1ΔIEC; Xbp1ΔIEC and Atg16l1ΔIEC; Xbp1ΔIEC colonies (adonis; Bray-Curtis; genotype, R² = 0.08782, P = 0.08192; gender, R² = 0.01233, P = 0.90809; litter, R² = 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; Atg16l1ΔIEC; Xbp1ΔIEC colony, R² = 0.7456, P = 0.435; Ern1<sup>−/−</sup>; Atg16l1ΔIEC; Xbp1ΔIEC colony, R² = 1.56, P = 0.178; Fig. 9 D). The results demonstrate that IRE1α-dependent development of ileitis is remarkably independent of the dysbiosis present in Atg16l1ΔIEC; Xbp1ΔIEC mice.

DISCUSSION

We report IRE1α 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 IRE1α caused by impairment of a selective autophagy process may be an important mechanism in how hypomorphic ATG16L1<sup>TRA10A</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 Nlpr6 mutants upon exposure to DSS (Elinav et al., 2011; Couturier-Maillard et al., 2013) or for ileitis in the Tnf<sup>TRA3E</sup> model (Schaubeck et al., 2016). Because alterations in the secretory compartment, lysozyme, and α-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...
Figure 5. Cross-compensation of IRE1α and IRE1β 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+ goblet cells in Em1iec−/−;Ern2−/− mice. n = 4/3/3/3/2. The mean ± 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−/− mice rederived germ free are protected from enteritis (Adolph et al., 2013) and do not accumulate Ire1α 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 pathobionts, e.g., as present in human’s real-life exposure, 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 pathobionts that can cause colitis, such as Helicobacter typhlonius in TRUC (Tbx21−/−; Rag2−/− 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−/− 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 (Lapasset 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 Ire1α 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 T300A carriers; the high risk allele frequency of ATG16L1T300A in itself already argues against such a simple relationship (Jostins et al., 2012).

The continual accumulation of stress hits over time that lead to Ire1α engagement may at some point overwhelm the epithelium’s capacity to resolve ER stress, whereby hypomorphic ATG16L1 T300A 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). Notably, high fat diet and obesity, the latter associated with markedly increased risk for developing CD (Khaliﬁ 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 Ire1α via the Sel1L–Hrd1 ER-associated degradation complex under basal conditions (Sun et al., 2015). In contrast, Sel1L-dependent Ire1α 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 Ire1α dimerization, oligomerization, and higher-order cluster formation are associated with escalating states of Ire1α activation and a transition from homeostatic to pro-death and proinflammatory UPR (Korenykh 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 Ire1α. Ubiquitination of Ire1α has been associated with both Ire1α’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 Ire1α levels (not depicted), indicating that autophagy-dependent Ire1α removal may be selective and regulated differently from the bulk process of ER–phagy.

The specific role for the unique expression of both Ire1α and Ire1β in the intestinal epithelium has remained enigmatic. A clue comes from Ern2−/− 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, Ire1α and Ire1β have divergent roles in organ inﬂammation. This feature is conspicuous in the Atg16l1−/−Xbp1−/− mouse line, which has a protective role in the intestines regarding the development of ileitis (Bertolotti et al., 2001). In the current study, we sought to investigate whether such a protective role also exists in ileitis. In order to determine whether the protective role of Xbp1−/− mice is due to a decreased expression of FAM134b, we measured the mRNA levels of Xbp1−/− in ileitis. The first panel shows that the expression of FAM134b is decreased in ileitis compared to wild-type mice. This indicates that the protective role of Xbp1−/− mice is not due to a decreased expression of FAM134b.
Figure 6. Selective autophagy receptors target IRE1α 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 ± SEM is shown. One-way ANOVA with Bonferroni’s correction was used. (B) IRE1α immunoblot (IB) of Xbp1-sufficient (MODE-K/iCtrl) and Xbp1-deficient (MODE-K/iXbp1) MODE-K cells co-cultured for 24 h with bafilomycin (Baf), an inhibitor of vacuolar H⁺ ATPase that prevents cargo deggregation in autophagolysosomes. Data are representative of three independent experiments. (C) IRE1α 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 IRE1α with selective
hyperactive IRE1α 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 IRE1α as driver of ileal inflammation. Correspondingly, we show that ATG16L1T300A-related genetic risk associates with IRE1α 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., 2001), links IRE1α 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 IRE1α 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;Xbp1ΔIEC (Xbp1ΔIEC), Villin-Cre;Atg16l1ΔIEC (Atg16l1ΔIEC), Villin-Cre;Atg16l1ΔIEC;Xbp1ΔIEC (Atg16l1ΔIEC;Xbp1ΔIEC), Villin-Cre;Ern1ΔIEC (Ern1ΔIEC), Ern2ΔIEC, and Villin-Cre;Ern1ΔIEC;Xbp1ΔIEC mice have been described previously (Bertolotti et al., 2001; Kaser et al., 2008; Adolph et al., 2013; Niederreiter et al., 2013). Villin-Cre;Atg16l1ΔIEC;Ern1ΔIEC, Villin-Cre;Xbp1ΔIEC;Ern2ΔIEC (Xbp1ΔIEC;Ern2ΔIEC), Villin-Cre;Atg16l1ΔIEC;Xbp1ΔIEC;Ern1ΔIEC (Atg16l1ΔIEC;Xbp1ΔIEC;Ern1ΔIEC), Villin-Cre;Atg16l1ΔIEC;Ern1ΔIEC;Xbp1ΔIEC;Ern2ΔIEC (Atg16l1ΔIEC;Ern1ΔIEC;Ern2ΔIEC), Villin-Cre;Ern1ΔIEC (Ern1ΔIEC), Villin-Cre;Ern1ΔIEC;Ern2ΔIEC (Ern1ΔIEC;Ern2ΔIEC), and Villin-Cre;Ern1ΔIEC;Xbp1ΔIEC;Ern2ΔIEC (Ern1ΔIEC;Xbp1ΔIEC;Ern2ΔIEC) mice were generated by intercrossing the relevant individual strains. All mice were on a C57BL/6 background except Ern1ΔIEC; Ern2ΔIEC mice. n = 3. Data are representative of two independent experiments. (F) Coimmunoprecipitation of IRE1α with OPTN in MODE-K.iCtrl and MODE-K.iXbp1 cells. n = 2. Data are representative of two independent experiments. Ctrl, control.

Antibodies and reagents
The following antibodies were used for immunoblotting and immunoprecipitation: anti-IRE1β (Bertolotti et al., 2001), anti–phospho-IRE1β (gift from K. Kohn, Nara Institute of Science and Technology, Takayama, Ikoma, Nara, Japan), anti-IRE1α (20790; H–190; polyclonal; Santa Cruz Biotechnology, Inc.), anti-IRE1α (3294; 14C10), anti–NPD52 (9036; polyclonal; anti-NBRI (9891; D2E6), anti–GAPDH (2118; 14C10), anti–β-actin (4970; 13E5; Cell Signaling Technology), antiantiuretin (10000; polyclonal; Cayman), and anti–p62 (PM045; polyclonal; MBL). Immunofluorescence antibodies were anti-IRE1α (20790; H–190), antilysozyme (27958; C19), anti-MUC2 (15334; H–300), anti-IRE1β (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; ab96931; polyclonal), donkey anti–goat IgG (DyLight 488; ab96931; polyclonal), donkey anti–rabbit IgG (DyLight 650; ab96894; polyclonal; Abcam), and CleanBlot (21230; Thermofisher scientific). UEA-1 (RL-1062; Vector Laboratories) was used to stain secretory IECs.
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;Xbp1ΔIEC mice predisposes to colitis but not ileitis. (A) Cross-fostering of Wt (V-cre− Atg16l1fl/fl;Xbp1fl/fl) offspring derived from a Wt (V-cre− Atg16l1fl/fl;Xbp1fl/fl) breeding pair to a Wt (V-cre− Atg16l1fl/fl;Xbp1fl/fl) foster dam and vice versa. Male breeders of the F0 generation were removed from the cage before F1 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− Atg16l1fl/fl;Xbp1fl/fl; displayed in black) and Atg16l1;Xbp1ΔIEC (DKO; V-cre+ Atg16l1fl/fl;Xbp1fl/fl; displayed in shaded red) offspring derived from a hemizygous breeding pair (V-cre− Atg16l1fl/fl;Xbp1fl/fl [Wt] × V-cre+ Atg16l1fl/fl;Xbp1fl/fl [DKO]) from a Wt (V-cre− Atg16l1fl/fl;Xbp1fl/fl) dam (biological mother; BM) to a Atg16l1;Xbp1ΔIEC (DKO; V-cre− Atg16l1fl/fl;Xbp1fl/fl) foster mother (FM; displayed in red). Male breeders of the F0 generation were removed from the cage before F1 offspring was born (displayed in shaded red). Before weaning of cross-fostered offspring, pups were genotyped, and Atg16l1;Xbp1ΔIEC littermates were removed from the cage. Remaining Wt offspring that acquired Atg16l1;Xbp1ΔIEC 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 offspring that acquired Atg16l1;Xbp1ΔIEC 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). (D) 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;Xbp1ΔIEC 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 = 5/6/6/6/4/7. The median is shown. (G, left) Cross-fostering of either Atg16l1ΔIEC offspring (KO; displayed in blue) and their Wt littermates (displayed in yellow) or Xbp1ΔIEC offspring (KO; displayed in blue) and their Wt littermates (displayed in yellow) to an Atg16l1;Xbp1ΔIEC (DKO; displayed in red) foster dam allowing them to acquire the Atg16l1;Xbp1ΔIEC 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 Ribosomal 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 with a 97% similarity cut off. R software was used to analyze α and β 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) and 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.

**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.

**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+ 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+ 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 1 h at subboiling temperature. 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+ 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+ 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.
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. 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...
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′-TTGAGTCCAAGGTGTTAG-3′; and reverse, 5′-CAGTAGCTGTACCCTCACTTC-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 siRNA 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), 5 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 OptiMEM (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′-GGGGAATTCAGGTGAAG-3′ (Amthor et al., 2009); and mHprt, 5′-GTGAACATCGAGCCCAAC-3′ and 5′-AGGGCATATCCAAACAACTTT-3′ (Amthor et al., 2009). Target gene expression was normalized to housekeeping 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 (HotStarTag; QIAGEN) using the primers mXbp1 sp. forward 5′-ACACGCTTGGGAGTGGACAC-3′ and mXbp1 sp. reverse 5′-CCATGGGAAGATGTTCTG GG-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 IRE1α 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 performed and analyzed most experiments. R. Bharti and P. Rosenstiel performed and analyzed 16S rDNA sequencing experiments. M. Parkes supervised collection of human biopsies. M. Tschurtschenthaler, J.W. Ashcroft, T.E. Adolph, and L. Niederreiter to together with S. Saveljeva, J. Bhattacharyya, M.B. Flak, and G.M. Fuhler performed and analyzed new mutant mouse strains. G.M. Fuhler, C.J. van der Woude, and M.P. Peppelenbosch contributed to R.S. Blumberg.

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