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## Role of endoplasmic reticulum stress and autophagy as interlinking pathways in the pathogenesis of inflammatory bowel disease

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### Abstract

**Purpose of review**—The purpose of this study is to provide an overview of the role of endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) in inflammatory bowel disease (IBD).

**Recent findings**—Human genetic studies have identified several UPR-related genes and autophagy-related genes as IBD risk loci. Impairment of each branch of the UPR causes spontaneous enteritis or creates higher susceptibility for intestinal inflammation in model systems. Deficiency of either UPR or autophagy in small intestinal epithelial cells promotes each other's compensatory engagement, which is especially prominent in Paneth cells such that, in the absence of both, severe spontaneous enteritis emerges.

**Summary**—Interactions between the UPR and autophagy exhibit critical synergistic interactions within the intestinal epithelium and especially Paneth cells that are of considerable importance to the maintenance of homeostasis. When dysfunctional in the Paneth cell, spontaneous inflammation can emerge that may extend beyond the epithelium providing direct experimental evidence that subsets of Crohn's disease may emanate from primary Paneth cell disturbances.

### Keywords

autophagy; Crohn's disease; endoplasmic reticulum stress; inflammatory bowel disease; Paneth cells

## INTRODUCTION

The intestinal epithelium consists of six major cell types that are derived from the common stem cell: Paneth cells, goblet cells, tuft cells, microfold villus (M) cells, absorptive cells

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### Conflicts of interest

The authors declare no conflicts of interest to disclose.

and enteroendocrine cells. These together form a single layer of cells that line the gastrointestinal tract and represent a critical interface between the host and enteric microorganisms. Paneth cells, which are primarily located at the base of small intestinal crypts under homeostatic conditions, secrete multiple antimicrobial peptides (AMPs) including defensins and lysozyme and thereby regulate the composition of the host commensal microbiota as well as play an important role in innate immunity and the function of the stem cell niche [1,2]. Goblet cells secrete abundant amounts of mucins, which constitute the mucus barrier and enteroendocrine cells produce significant levels of neuropeptides [3,4]. Further, the vast majority of absorptive epithelial cells also broadly regulate the composition of the commensal microbiota and subjacent mucosal immune system via secretion of a large array of cytokines and chemokines, which makes them an important component of the mucosal immune system [5]. These activities of intestinal epithelial cells (IECs) create a large secretory burden for this cell type and it demands appropriate cell biologic systems to manage this.

The unfolded protein response (UPR), in particular, is important for the development and survival of highly secretory cells such as IECs that synthesize high volumes of proteins, which requires careful management of the endoplasmic reticulum (ER) to avoid stress in this organelle [6]. As Paneth and goblet cells are among the most secretory of the IEC subtypes, these cells are particularly prone to abnormalities when the UPR is dysfunctional [7,8]. Consistent with this, the small intestine, which contains high numbers of Paneth cells, has higher baseline levels of UPR-related gene expression than the colon [9]. The UPR is not only of importance to the function of differentiated IEC subtypes but also gut stem cells in general as ER stress and UPR induction are critical to such cells in the intestine [10] and oesophagus [11]. Given these broad biologic effects and evidence that UPR-related genetic risk loci are associated with inflammatory bowel disease (IBD), it is important to understand how these pathways operate in the maintenance of intestinal homeostasis and disease.

## THE UNFOLDED PROTEIN RESPONSE

ER stress is caused by the accumulation of unfolded and misfolded proteins in the ER arising from either primary (genetic) or secondary (environmental) factors [12,13]. Highly secretory cells, such as plasma cells, insulin-secreting  $\beta$ -cells in the pancreas, IECs, (especially Paneth cells and goblet cells) are highly susceptible to ER stress [6]. The UPR is a highly conserved mechanism that enables the cell to respond to and resolve the ER stress. The UPR is characterized by three major response arms. Specifically, ER stress triggers the activity of three ER membrane resident proteins that sense the presence of unfolded proteins in the ER lumen: inositol-requiring transmembrane kinase endonuclease 1 (IRE1), pancreatic ER kinase (PERK) and activated transcription factor 6 (ATF6). IRE1, PERK and ATF6 exist as inactive complexes in association with immunoglobulin heavy chain binding protein (BIP), which is also known as glucose-regulated protein 78 (GRP78), in the absence of misfolded proteins in the ER lumen. BIP senses the presence of misfolded proteins and releases the three proteins to allow them to enter their active states. IRE1 is the most evolutionarily conserved pathway and is shared by yeast [14,15]. Mammalian cells have two highly conserved isoforms, IRE1 $\alpha$  that is expressed ubiquitously [16] and IRE1 $\beta$  that is restricted to gut and lung epithelial cells [17]. IRE1 is dimerized and phosphorylated under

ER stress. The activated IRE1 has both kinase activity that promotes activation of JUN N-terminal kinase (JNK) [18] and Nuclear factor- $\kappa$ B (NF- $\kappa$ B) [19] and endoribonuclease activity that excises a 26-nucleotide sequence of X-box binding protein 1 (*XBPI*) mRNA resulting in a frame shift and generation of a transcriptionally active isoform that functions as a transactivator of UPR target genes [20]. Such target genes play a role in expanding the ER, for example, to allow the cell an ability to deal with its increased secretory requirements and diminish the ER stress. In addition, IRE1 mediates the rapid degradation of a certain subset of mRNAs, a process that is termed RIDD (regulated IRE1-dependent decay) [21]. PERK possesses protein kinase activity and inactivates elongation initiation factor 2 $\alpha$  (eIF2 $\alpha$ ) by phosphorylation that halts general protein synthesis but allows the translation of specific species of mRNA such as activating factor 4 (ATF4), which is translocated into the nucleus and activates genes necessary for the UPR. Finally, upon dissociation from GRP78, ATF6 translocates to the Golgi whereupon it is cleaved by site 1 and site 2 proteases (S1P and S2P) releasing the cytoplasmic domain of ATF6 (ATF6-N), which is transcriptionally active in inducing UPR-associated genes. ATF4 and the ATF6-N are critical to the activation of the apoptosis-related transcription factors, such as CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) that leads to cell cycle arrest and/or apoptosis [22,23\*]. Thus, if ER stress is unresolved, cell death is the likely outcome (Fig. 1).

## INOSITOL-REQUIRING TRANSMEMBRANE KINASE ENDONUCLEASE 1 – XBP1 BRANCH AND INTESTINAL INFLAMMATION

Several studies have shown that genetic deletion of molecules involved in the UPR are associated with either spontaneous intestinal inflammation and/or increased sensitivity to the experimental induction of colitis. IRE1 $\beta$  knockout mice are highly sensitive to dextran sodium sulphate (DSS)-induced colitis [24]. IRE1 $\beta$ -deficient mice exhibit increased basal levels of GRP78 in the intestinal epithelium prior to colitis induction suggesting ongoing ER stress and increased sensitivity to environmental agents that disrupt the IEC barrier and/or induce further ER stress [24]. IRE1 $\beta$  is particularly active in goblet cells such that IRE1 $\beta$  knockout (*Ern2*<sup>-/-</sup>) mice exhibit aberrant mucin 2 (MUC2) accumulation in the ER of goblet cells in contrast to IRE1 $\alpha$  knockout (*Ern1*-*IEC*) mice that have normal goblet cells [25\*]. This suggests that the sensitivity to DSS-induced colitis in *Ern2*<sup>-/-</sup> mice might be due to associated abnormalities in goblet cells in this model.

Mice with conditional deletion of XBP1 expression in IECs (*Xbp1*<sup>IEC</sup> mice) have been observed to develop spontaneous inflammation in the small intestine with microscopic features that include crypt abscesses, mononuclear and polymorphonuclear cell infiltration and ulcerations that mimic certain features of human IBD [7]. *Xbp1*<sup>IEC</sup> mice exhibit increased ER stress in the small intestine as detected by increased GRP78. As a consequence, IECs of *Xbp1*<sup>IEC</sup> mice exhibit increased apoptosis and a regenerative response, resulting in a reduction of goblet cells and Paneth cells with a condensed ER and absence of their characteristic secretory granules, in association with decreased AMP production. Consistent with a defect in Paneth cells, *Xbp1*<sup>IEC</sup> mice, such as *Nod2*<sup>-/-</sup> mice [26], are highly susceptible to infection with *Listeria monocytogenes* [7]. Although *Xbp1*<sup>IEC</sup> mice do not exhibit spontaneous colitis, they are highly susceptible to DSS-

induced colitis [7]. The mechanistic basis for this disparity between susceptibility to inflammation in the colon and small intestine in the presence of XBP1-deficiency and ER stress is not known. It has been pointed out that increased basal ER stress may exist in the small intestine [9]. It is also possible that the major effect of ER stress on Paneth cells may result in bacterial dysbiosis [7]. Both mechanisms are possible, as the spontaneous enteritis observed in *Xbp1*<sup>IEC</sup> mice can be significantly reversed by rederivation under germ-free conditions [27<sup>\*\*</sup>]. Together, these studies show that when IECs are unable to properly manage ER stress, not only can spontaneous intestinal inflammation emerge in the small intestine, but also the intestine at large is exquisitely sensitive to environmental factors capable of triggering intestinal inflammation.

The kinase activity of IRE1 has been linked to the activation of NF- $\kappa$ B and JNK, both of which are critical molecules in intestinal inflammation [28]. Consistent with this, mechanistic studies have shown that IRE1-dependent NF- $\kappa$ B and JNK activation is increased in XBP1-deficient IECs *in vivo*. Further, the NF- $\kappa$ B hyperactivation observed in *Xbp1*<sup>IEC</sup> mice is derived from increased IRE1 $\alpha$  activation and responsible for the intestinal inflammation that is observed. Thus, small molecule blockade of NF- $\kappa$ B activation or genetic deletion *Ern1* in IECs protects *Xbp1*<sup>IEC</sup> mice from spontaneous enteritis. Further, consistent with the important role played by tumour necrosis factor- $\alpha$  (TNF $\alpha$ ) in intestinal inflammation, *Xbp1*<sup>IEC</sup> mice are protected from enteritis upon deletion of TNF-receptor 1 [27<sup>\*\*</sup>]. These studies indicate that the development of enteritis on *Xbp1*<sup>IEC</sup> mice depends upon IRE1-NF- $\kappa$ B signalling in a pathway that is driven by TNF $\alpha$ , the cytokine targeted by the most potent therapeutics of human Crohn's disease [29].

The IRE1 and XBP1 pathway also has an important role in tumourigenesis. *Xbp1*<sup>IEC</sup> mice exhibit increased numbers of Lgr5<sup>+</sup> and Olfm4<sup>+</sup> intestinal stem cells (ISCs), which is dependent upon *Ire1a* [30], and a Stat3-mediated increase in the proliferative output of transit-amplifying cells [31<sup>\*</sup>], the latter being dependent upon inflammatory cytokines produced by the epithelium that is undergoing ER stress. It was specifically observed that colitis-associated cancer induced by azoxymethane (AOM) followed by DSS resulted in 10-fold more and larger colonic tumours in *Xbp1*<sup>IEC</sup> mice than that observed in control mice. In addition, when *Xbp1*<sup>IEC</sup> mice were crossed onto an *Apc*<sup>min</sup> background (*Xbp1*<sup>IEC</sup>; *Apc*<sup>min</sup>), they developed twofold more tumours than their *Xbp1*<sup>Wt</sup> *Apc*<sup>min</sup> littermates. Further evidence that ER stress may promote intestinal neoplasia is evidence that stem cells undergoing ER stress rapidly enter the transit-amplifying compartment and undergo significant proliferation [31<sup>\*</sup>].

## OTHER ENDOPLASMIC RETICULUM STRESS RELATED MOLECULES AND INTESTINAL INFLAMMATION

Mice with deficiency in ATF6 $\alpha$  (*Atf6a*<sup>-/-</sup> mice) are sensitive to DSS-induced colitis with evidence of increased ER stress, such as increased expression of GRP78, ATF4, CHOP and spliced *Xbp1* [32<sup>\*</sup>]. *P58*<sup>IPK</sup><sup>-/-</sup> mice, which are deficient in this important ER chaperone, have also been demonstrated to be sensitive to DSS-induced colitis with increased GRP78 and CHOP expression and phosphorylation of IRE1. Mice with conditional ablation of eIF2 $\alpha$  phosphorylation exhibit defective UPR signalling and ER-associated mRNA

translation, leading to protein secretion dysfunction in Paneth cell and increased sensitivity to *Salmonella* infection and DSS-induced colitis [33\*].

Anterior gradient 2 (AGR2), which is a member of the ER protein disulfide isomerase (PDI) gene family, is expressed in goblet, Paneth and enter-oendocrine cells in the small intestine [34,35]. Zhao *et al.* [36] have reported that *Agr2*<sup>-/-</sup> mice are observed to develop spontaneous ileocolitis with increased levels of ER stress markers; XBP1s, CHOP, PERK and GRP78 (BiP). These mice have decreased Mucin 2 expression in goblet cells and abnormal localization and expansion of Paneth cells in the small intestine. On the contrary, *Chop*<sup>-/-</sup> MEFs are resistant to ER stress induced apoptosis [37] and *Chop*<sup>-/-</sup> mice are protected from DSS colitis and colitis induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS) [38]. CHOP may therefore be an interesting therapeutic target in the treatment of IBD.

Forward genetic approaches using N-ethyl-N-nitrosourea mutagenesis have resulted in several new mouse models that exhibit defects in the UPR. For example, mice with two distinct single missense mutations in *Muc2* (*Winnie* and *Eeyore*) have been reported to develop spontaneous colitis resembling human ulcerative colitis [39]. These mutations cause mucin misfolding leading to accumulation of MUC2 precursor proteins in the ER, ER vacuolization and ER stress confirmed by increased levels of *Xbp1* splicing and GRP78 [39]. The inflammatory response in this model involves both innate and adaptive immunity including the interleukin (IL)-23/Th17 inflammatory responses, as observed in human IBD [40]. Another mouse model, so-called *woodrat* mouse, possesses a loss-of-function mutation in site-specific protease 1 (S1P). S1P and S2P are responsible for the generation of the transcriptionally active form of ATF6-N through release of the cytoplasmic tail of ATF6 when it is translocated to the Golgi apparatus during ER stress [41]. The *woodrat* mouse exhibits increased sensitivity to DSS-induced colitis with the defect largely derived from the radio-resistant compartment in bone marrow chimera experiments pointing towards the intestinal epithelium as the likely source of the defect. One caveat of this model is due to the broad substrate specificity of S1P that includes ATF6, CREB-H and other related transcription factors [42]; hence, it cannot be concluded that the phenotype observed is directly due to ATF6.

OASIS, which is a basic leucine zipper transmembrane transcription factor activated in response to ER stress, is expressed in goblet cells of the large intestine. *Oasis*<sup>-/-</sup> mice have abnormal differentiation and maturation of goblet cell [43], resulting in increased susceptibility to DSS-induced colitis [44].

In summary, a large variety of defects in the UPR that result in ER stress render the intestinal epithelium susceptible to the development of spontaneous or induced enterocolitis. The determination of whether spontaneous or induced disease develops likely reflects the depth of the effect that the mutation has on the UPR and the susceptibility thereby to environmental stresses. In defects associated with *Xbp1* or *Agr2*, for example, the pressure derived from the commensal microbiota *per se* is enough to tip the host over to spontaneous intestinal inflammation. Another aspect of these studies is greater apparent sensitivity of the small relative to the large intestine to the effects of ER stress. This is further buttressed by the exquisite sensitivity of the small intestine to the effects of ischemia/reperfusion (I/R)

injury and implies that loss of Paneth cells due to ER stress is crucial for I/R-related disease [45]. I/R of the human small intestine has been shown to activate the UPR by evidence of increased splicing of *XBPI* and increased BiP, CHOP and GADD34.

## A ROLE OF AUTOPHAGY ON ENDOPLASMIC RETICULUM STRESS RELATED INFLAMMATION

Autophagy is an evolutionarily conserved and fundamental cellular mechanism that orchestrates the degradation of proteins, lipids and organelles in order to maintain homeostatic function. Autophagy is activated in response to multiple stresses that include processes such as hypoxia, infection, nutrient starvation and ER stress [46,47]. Autophagy is either considered as macroautophagy or microautophagy with the latter being directed at specific organelles [48]. Macroautophagy in response to pathogens is considered xenophagy.

Human genetic studies have revealed that autophagy is a major mechanism in the pathogenesis of Crohn's disease through the discovery of autophagy-related 16-like 1 (*ATG16L1*) as a major genetic risk factor [49]. This has been significantly buttressed by the identification of further genetic defects that also support defects in autophagy as being critical to Crohn's disease pathogenesis. These include genes such as nucleotide-binding oligomerization domain-containing protein 2 (*NOD2*), leucine-rich repeat serine/threonine-protein kinase 2 (*LRKK2*) and immunity-related GTPase M (*IRGM*), which have been identified as genetic risk factors for IBD [50].

Most attention in this area has been on *ATG16L1*, which is one of the strongest risk factors in Crohn's disease. The majority of genetic risk related to *ATG16L1* is derived from a single non-synonymous single nucleotide polymorphism (SNP) that encodes for a single nonsynonymous amino acid substitution (Thr300Ala) [49]. This has been modelled in mice that express hypomorphic *ATG16L1* function, so-called *Atg16l1* hypomorphic mice (*Atg16l1<sup>HM</sup>*). Such mice do not develop spontaneous intestinal inflammation but exhibit morphologic and functional evidence of Paneth cell dysfunction as do humans who are homozygous for the Thr300Ala allele [51]. The morphologic defect observed is notable for Paneth cells with decreased quantities of intracellular granules and of reduced size together with evidence of increased pro-inflammatory mediator production such as serum amyloid A [52]. More recently, a functional mechanism of Thr300Ala variant has been unveiled using Thr300Ala knock-in mice (Thr316Ala in mice), which have defective clearance of *Yersinia enterocolitica* and an elevated inflammatory cytokine response. The variant increases *ATG16L1* sensitization to caspase-3-mediated degradation, resulting in diminished autophagy and pathogen clearance [53]. Given that autophagy is highly active in Paneth cells [27], and recent evidence that Paneth cell autophagy plays an important compensatory role in the context of ER stress, it is likely that environmental factors that induce ER stress are particularly problematic for the host that is unable to mount an effective autophagic response. Specifically, it is well known that the UPR in response to ER stress is a major inducer of autophagy [46,54–56]. In the presence of unresolved ER stress in the intestinal epithelium as caused by *XBPI*-deficiency or potentially yet-to-be defined environmental factors, the inability to effectively engage autophagy due to genetic deficiency in autophagy as modelled by *ATG16L1* or *ATG7*-deficiency is associated with severe spontaneous

enteritis that is able to extend transmurally when both pathways are dysfunctional [27<sup>\*\*\*</sup>]. It is notable that the superficial spontaneous enteritis associated with intestinal epithelial deficiency of XBP1 is converted into an inflammatory response that extends into the submucosa when autophagy is disabled. Interestingly, the mechanistic link between the UPR and autophagy induction in the intestinal epithelium is mainly derived from the PERK-eIF2 $\alpha$ -ATF4 pathway that may be amenable to pharmacologic manipulation. Perhaps most importantly, these pathways are most active in Paneth cells such that specific deletion of genes in this cell type such as *Xbp1* and *Atg7* partially phenocopies what is observed when these genetic targets are extinguished throughout the intestinal epithelium [57<sup>\*\*\*</sup>]. Thus, mouse model systems suggest that intestinal inflammation associated Crohn's disease can emerge primarily from the intestinal epithelium and the Paneth cell in particular, suggesting that a subset of small intestinal Crohn's disease might be a specific disorder of the Paneth cell and potentially other IEC subtypes. As increased GRP78 and pEIF2 $\alpha$  has been detected in Paneth cells in patients with Crohn's disease who possess the *ATG16L1*<sup>T300A</sup> risk allele and in the setting of small intestinal disease, it is possible that these observations are extensible to humans [57<sup>\*\*\*</sup>].

## ENDOPLASMIC RETICULUM STRESS, UNFOLDED PROTEIN RESPONSE AND HUMAN GASTROINTESTINAL DISEASE

Human genetic research of IBD has identified several UPR-related genes that are associated with Crohn's disease and ulcerative colitis. Candidate gene approaches for IBD have identified *AGR2* and *XBP1* as risk loci associated with Crohn's disease and ulcerative colitis. Regarding the former, *Agr2*<sup>-/-</sup> mice develop spontaneous ileocolitis with ER stress as described above. Genetic linkage studies from three independent microsatellite-based genome scans revealed a locus on chromosome 22 close to the *XBP1* gene as a risk locus for Crohn's disease and ulcerative colitis. Deep sequencing for all exons, splice sites and promoter regions of *XBP1* gene revealed that several rare variants including two hypomorphic variants are more frequently present in the Crohn's disease and ulcerative colitis than in the control cohort [7]. Genome-wide association studies found Orosomucoid-like 3 (*ORMDL3*), which is involved in ER calcium homeostasis, as the risk for Crohn's disease [58] and ulcerative colitis [59].

Several recent studies have shown evidence for increased ER stress in the intestines of humans with Crohn's disease and ulcerative colitis. For example, GRP78 expression and *XBP1* splicing have been observed to be increased in the small intestine and colon of Crohn's disease patients relative to that observed in healthy controls [7,60,61]. In ulcerative colitis, GRP78 expression is increased in the colon, especially in inflamed mucosa [7,39,60]. Treton *et al.* [62] have specifically reported higher expression levels of unspliced and spliced *XBP1*, GRP78, GRP94 and ER degradation enhancer mannosidase alpha-like 1 (EDEMI) in colonic mucosa in association with increased levels of the active form of ATF6 (ATF6 $\alpha$ -p50) together with an impairment of the eIF2 $\alpha$ -ATF4-CHOP pathway in ulcerative colitis. In contrast, Hu *et al.* [63] reported increased p-eIF2 $\alpha$  in the colonic mucosa of Crohn's disease and ulcerative colitis patients. Although these studies need to be reconciled, they suggest that in ulcerative colitis, and potentially Crohn's disease, an active UPR may be

uncoupled from autophagy induction that would be predicted to promote intestinal inflammation.

## CONCLUSION

The IEC is highly susceptible to ER stress, which is likely due to the unique environmental conditions within which the IEC must function. As such, the IEC is highly dependent upon the UPR for the maintenance of homeostasis that assists in expanding the capabilities of the ER and proteostasis. In addition, the UPR critically engages autophagy to compensate for ER stress in the intestinal epithelium. When either the UPR and/or autophagy are disabled due to genetic and/or environmental factors, intestinal inflammation can emerge. Genetic factors that affect the UPR include those associated with *XBPI*, *AGR2* and potentially *ORMDL3*, whereas those that affect autophagy include *ATG16L1*, *IRGM* and potentially *LRKK2*. Interestingly, *IRGM* defects have recently been shown to be associated with defects in Paneth cells [64]. Environmental factors that affect the UPR include factors such as hypoxia, microbial toxins (e.g. Shiga toxicogenic factors that degrade GRP78) and dietary factors (e.g. iron) [65,12]. Similarly, environmental factors that potentially affect autophagy include infectious pathogens that activate intracellular caspases such as caspase 3 to which the Thr300Ala allele of *ATG16L1* is highly susceptible and cleaved into an inactive state [53]. Not only do such insights shed light on our understanding of the pathogenesis of IBD but also may lead to new therapeutic strategies such as the use of chemical chaperones to enhance the UPR, mammalian target of rapamycin (mTOR) blockers to increase autophagy or inhibitors of activated IRE1a or CHOP to diminish a pathogenic UPR.

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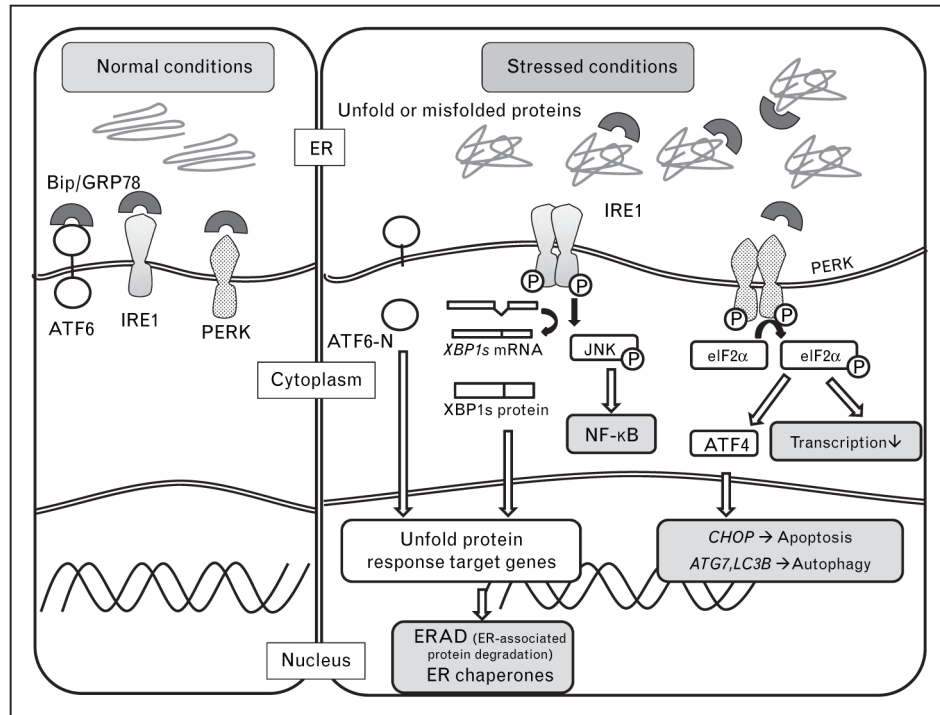
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**KEY POINTS**

- Several UPR-related genes and autophagy-related genes have been identified as inflammatory bowel disease risk loci.
- Genetic and environmental factors induce ER stress in the intestinal epithelium and especially Paneth cells.
- Autophagy is induced by ER stress and plays an important compensatory role.
- Uncompensated ER stress due to autophagy defects promotes small intestinal and likely large intestinal inflammation.
- Intestinal inflammation can emerge directly from the intestinal epithelium and the Paneth cell in particular.



**FIGURE 1.** Endoplasmic reticulum stress inducing autophagy. BIP (GRP78) senses the presence of misfolded proteins and releases ATF6, IRE1 and PERK to allow them to enter their active states, resulting in transcriptional programs that relieve the ER stress and activate autophagy. ATF4, downstream of the PERK-eIF $\alpha$  pathway, transactivates *LC3b* and *Atg7*, resulting in activation of the compensatory autophagic mechanism.