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REVIEW

The unfolded protein response and gastrointestinal disease

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Abstract As the inner lining of the gastrointestinal tract, the intestinal epithelium serves an essential role in innate immune function at the interface between the host and microbiota. Given the unique environmental challenges and thus physiologic secretory functions of this surface, it is exquisitely sensitive to perturbations that affect its capacity to resolve endoplasmic reticulum (ER) stress. Genetic deletion of factors involved in the unfolded protein response (UPR), which functions in the resolution of ER stress that arises from misfolded proteins, result in spontaneous intestinal inflammation closely mimicking human inflammatory bowel disease (IBD). This is demonstrated by observations wherein deletion of genes such as *Xbp1* and *Agr2* profoundly affects the intestinal epithelium and results in spontaneous intestinal inflammation. Moreover, both genes, along with others (e.g., *ORDML3*) represent genetic risk factors for human IBD, both Crohn's disease and ulcerative colitis. Here, we review the current mechanistic understanding for how unresolved ER stress can lead to intestinal inflammation and highlight the findings that implicate ER stress as a genetically affected biological pathway in IBD. We further discuss environmental and microbial factors that might impact on the epithelium's capacity to resolve ER stress and which may constitute exogenous factors that may precipitate disease in genetically susceptible individuals.

Keywords Unfolded protein response (UPR) · Endoplasmic reticulum (ER) stress · Inflammatory bowel disease (IBD)

Abbreviations

ABCG2	ATP-binding cassette G2
AIDS	Acquired immunodeficiency syndrome
AIEC	Adherent-invasive *Escherichia coli*
AGR2	Anterior gradient 2
ASK1	Apoptosis signal-regulating kinase 1
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
BAK	BCL2-antagonist/killer 1
BAX	BCL2-associated X protein
BBF2H7	Box B-binding factor 2 human homolog on chromosome 7
BiP	Binding immunoglobulin protein
CD	Crohn's disease
CEACAM6	Carcinoembryonic antigen-related cell adhesion molecule 6
CHOP	C/EBP homologous protein
CREB4	CRE-binding protein 4
CREBH	CRE-binding protein H
C/EBP	CCAAT/enhancer-binding protein
DSS	Dextran sodium sulfate
eIF2α	Elongation initiation factor 2α
ER	Endoplasmic reticulum
EDEM1	ER degradation enhancer, mannosidase alpha-like 1
ENU	*N*-ethyl-*N*-nitrosourea
ERAD	ER-associated degradation
GADD34	Growth arrest and DNA damage-inducible protein 34
grp78	78kDa glucose-regulated protein

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Grp94	Glucose regulated protein 94
HIV	Human immunodeficiency virus
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IRE1	Inositol-requiring enzyme 1
IEL	Intraepithelial lymphocyte
JNK	Jun N-terminal kinase
lZIP	Leucine zipper protein
MEF	Mouse embryonic fibroblast
MTP	Microsomal triglyceride transfer protein
MSI1	Musashi-1
OASIS	Old astrocyte specifically induced substance
ORMDL3	Orosomucoid-like 3
PERK	Protein kinase related (PKR)-like ER kinase
$P58^{IPK}$	Protein kinase inhibitor of 58 kDa
PBA	4-phenyl butyrate
RIDD	Regulated IRE1-dependent decay
S1P	Site-1 protease
S2P	Site-2 protease
SPF	Specific pathogen-free
SERCA2b	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2
SERCA	Sarco-endoplasmic reticulum Ca2+ pump
TNBS	Trinitrobenzene sulfonic acid
TNF	Tumor necrosis factor
TRAF2	TNF receptor associated factor 2
TNFR1	Tumor necrosis factor receptor type 1
TLR	Toll-like receptor
TUDCA	Tauro-ursodeoxycholic acid
UC	Ulcerative colitis
UDCA	Ursodeoxy-cholic acid
uORF	Upstream open reading frame
UTR	5' untranslated region
UPR	Unfolded protein response
VLDL	Very low density lipoprotein
XBP1	X-box binding protein-1

Introduction

The intestinal epithelium is the inner monolayer lining of the gastrointestinal tract which, from a teleological point of view, is the functional quintessence of the intestine as an organ. Intestinal epithelial cells (IECs) absorb nutrients and fulfill the remarkable task of separating the abundance of the commensal microbiota from the host environment [1]. Several types of IECs differentiate from a constantly replicating intestinal stem cell that has been well characterized in the small and the large intestine, and which forms the basis for the continuous replenishment of the intestinal epithelial surface every 48–72 h in rodents and 72–96 h in humans [2, 3]. The types of differentiated IECs are absorptive epithelial cells, which express a multitude of molecular transporters on their microvillous surface; enteroendocrine cells, which secrete hormones and other mediators that relay metabolic information to peripheral organs; Paneth cells at the base of small intestinal crypts (and analogous $CD24^+$ cells in colonic crypts [4, 5]), which provide the niche for intestinal stem cells, secrete abundant amounts of antimicrobial peptides that contribute to the host resistance to infection and contribute to the selective pressure imposed onto the intestinal microbial flora; and goblet cells, which secrete massive quantities of mucins, which constitute the highly organized and complex mucin layer which represents an integral key part of the intestinal barrier [2, 6]. In addition to these characteristic functions of the differentiated IEC types are their individual roles as central organizers of the mucosal immune system via secretion of key cytokines and chemokines [7], and in fact, the intestinal epithelium could be considered the most ancient part of the innate immune system [8]. In their entirety, covering 300 m^2 of intestinal surface area, IECs represent a massive, highly secretory organ that operates in a physiological environment quite adverse to the energy-dependent process of protein folding [9]. This includes an almost anaerobic milieu, high cellular turn-over, and exposure to microbial metabolites and toxins that may impact on protein folding. It is therefore not surprising that unimpaired handling of protein misfolding is critical for the physiological functions of IECs, and correspondingly, highly secretory Paneth and goblet cells appear most affected by impairment in UPR function [9].

ER stress elicits the UPR via three main ER transmembrane sensors which cooperate with the chaperone grp78 to sense misfolded proteins [10–12]: IRE1 (inositol-requiring enzyme 1), PERK (Protein Kinase Related-like ER kinase), and ATF6 (Activating transcription factor 6; α and β isoforms), amongst which IRE1 represents the evolutionarily most conserved branch of the UPR. The epithelium of the digestive and respiratory tracts are unique in that they express an additional isoform along with ubiquitously expressed IRE1α, namely IRE1β [13], which can be interpreted as an evolutionary hint toward the importance of ER stress sensing at mucosal barriers. Upon sensing of misfolded proteins, IRE1 is auto-phosphorylated via its kinase domain, forms dimers, and activates its endoribonuclease domain which results in unconventional splicing of *XBP1* (X-box binding protein-1) mRNA [14]. The excision of a 26-nt sequence of *XBP1* mRNA results in a frame-shift and the translation of spliced XBP1 (XBP1s), which functions as a potent transactivator of UPR target genes, cooperating with ATF6. In addition, protracted ER stress results in the activation of a further IRE1 endoribonuclease mechanism, termed RIDD (regulated IRE1-dependent decay) [15]. RIDD results in the selective degradation of ER-associated mRNAs, thereby decreasing the translational burden of the ER. ATF6, with its two isoforms α and β, is a type II ER transmembrane molecule that encodes a bZIP transcriptional activator in its cytoplasmic domain. Upon sensing misfolded proteins, ATF6

(or ATF6p90) is processed first via site-1 protease (S1P) followed by site-2 protease (S2P) within the Golgi apparatus, which releases its cytoplasmic domain (ATF6f or ATF6p50) that translocates to the nucleus and transactivates its target genes [10, 11]. Finally, PERK is a type I ER transmembrane protein kinase that, upon sensing ER stress, inhibits general protein translation into the ER through inactivation of the elongation initiation factor 2α (eIF2α) via serine phosphorylation. This translational attenuation helps alleviate ER stress. In addition to this function of PERK/eIF2α, eIF2α phosphorylation also leads to preferential increases in the translation of selective mRNAs which contain inhibitory upstream open reading frames (uORFs) within the 5' untranslated region which normally preclude their translation in the steady state [10, 11]. Via this mechanism, PERK/eIF2α activation results in the translation of activating transcription factor 4 (ATF4), which in turn upregulates a further subset of UPR genes. While ATF4 preferentially transactivates genes involved in amino acid transport, resistance to oxidative stress, and glutathione biosynthesis, ATF6f and XBP1s cooperate to transactive genes involved in ER-associated degradation (ERAD) and protein folding. XBP1s is further involved in transactivating genes important for protein entry into the ER, and via enzymes related to phospholipid biosynthesis, it is involved in the biogenesis of the ER and Golgi [10, 11]. Notably, XBP1s-regulated target genes can be separated into a group of core UPR genes that are shared among a variety of cell types and conditions and a remarkably diverse set of target genes that are transactivated in a cell type- and condition-dependent manner [16]. The underlying mechanism of this dichotomy is not entirely understood at the moment but might explain the remarkably different function of XBP1 in different cell types as revealed through cell-specific genetic ablation. In case these integrative adaptive mechanisms aimed at resolving ER stress fail, protracted and severe ER stress can induce apoptosis.

Mechanistic insight into the role of the UPR in gastrointestinal disease

IRE1/XBP1 signaling in IECs

At baseline, small and large intestinal IECs exhibit very little IRE1 activity as revealed through the presence of minute quantities of spliced XBP1 under homeostatic conditions [17–19]. A *Cre* reporter cloned in-frame with *Xbp1s*, whereby Cre recombinase activates a fluorescent reporter in cells with *Xbp1* splicing activity (i.e., IRE1 activation) and identified Paneth cells at the crypt base and small intestinal villus IECs as a site of active Xbp1 function [20].

The first insight into the importance of the IRE1/XBP1 branch of the UPR in IECs and the intestine in general came from studies of *Ire1*$\beta^{-/-}$ (*Ern2*$^{-/-}$) mice [13]. As noted above, Ire1β is specifically expressed in the intestinal and respiratory epithelium. Although *Ire1*$\beta^{-/-}$ mice do not exhibit any gross or microscopic anomalies, increased expression of grp78 (BiP) can be detected in epithelial cells of the stomach, small intestine, and colon in these mice, indicative of ER stress. This was shown to be associated with increased sensitivity to experimental colitis induced by the barrier disruptive reagent dextran sodium sulphate (DSS) (Fig. 1, Table 1).

Introduction of a premature stop codon close to the *N* terminus of XBP1, hence affecting XBP1u and XBP1s, by conditional deletion of only one allele of exon 2 of *Xbp1* in IECs via a Villin-promoter *Cre* transgene was sufficient to induce massive splicing of *Xbp1* mRNA (the splicing site is encoded from exon 4 and therefore retained for analysis) [18]. Along with the low abundance of XBP1s in the steady state, this indicates a very high sensitivity of IECs toward ER stress caused by even minor perturbations of XBP1 effector function. Although expression of the ER chaperone grp78 is to a substantial extent under the control of Xbp1, *Xbp1*$^{-/-(IEC)}$ mice nonetheless exhibited increased grp78 expression indicative of ER stress [18]. As a consequence of ER stress in IECs, mature Paneth cells were depleted. Remnant Paneth cells exhibited an absence of the typically expanded ER and only few and small granules compared to the extensive granule compartment observed on XBP1-sufficient Paneth cells. As a consequence, *Xbp1*$^{-/-(IEC)}$ mice possess decreased crypt bactericidal function and a consequent impairment in bacterial handling of model pathogens such as *Listeria monocytogenes* [18]. Similar cell biological alterations were also observed in goblet cells in *Xbp1*$^{-/-(IEC)}$ mice, together with an approximately 30 % reduction in their numbers. Furthermore, hypomorphic XBP1 function also resulted in an inflammatory hyperreactivity of IECs towards microbial (e.g., the TLR5 ligand flagellin) or cytokine (e.g., TNFα) stimuli, which was characterized by the induction of increased phosphorylation of c-Jun N-terminal kinase (JNK) and secretion of the neutrophil-attractant chemokine CXCL1 [18]. Increased apoptosis of the small intestinal epithelium as a consequence of unresolved ER stress in association with increased C/EBP homologous protein (CHOP; encoded by *Ddit3*) expression was further noted. These multiple consequences of XBP1 hypofunction in the intestinal epithelium are associated with the spontaneous development of small intestinal inflammation that is histologically similar to inflammatory bowel disease (IBD) in that it presents with crypt abscesses, neutrophil and mononuclear cell infiltration, and ulcerations [18]. While the colon does not exhibit spontaneous inflammation, *Xbp1*$^{-/-\ (IEC)}$ mice are more susceptible to DSS colitis compared to *Xbp1*$^{+/+(IEC)}$ littermates. Of note is that both spontaneous enteritis as

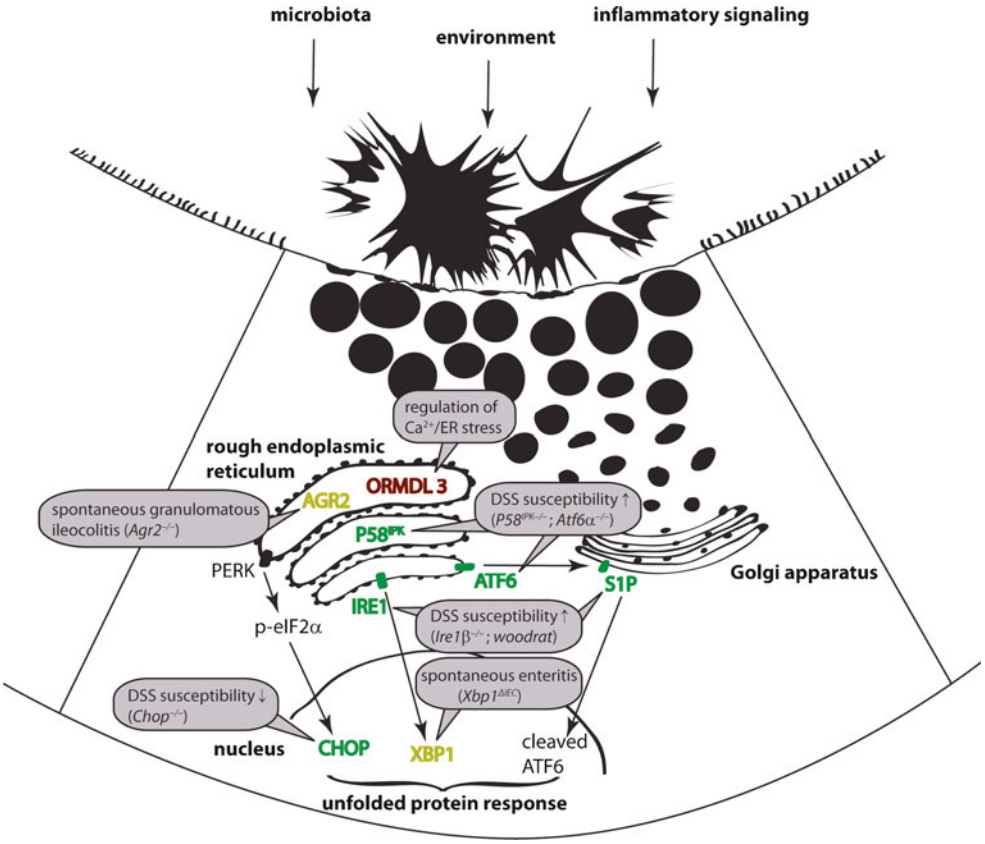

Fig. 1 The unfolded protein response and IBD. ER stress arises from host factors (e.g., genetic impairment of UPR-related genes or mutations that lead to protein misfolding; inflammatory signaling) or environmental factors (e.g., diet, microbial metabolites), and consequently activates the UPR via its three main branches PERK/eIF2α/ATF4; IRE1/XBP1; ATF6/ATF6f. Genetic targeting or ENU-induced mutagenesis revealed the importance of the individual gene products for mucosal homeostasis through animal models as indicated. Several genes involved in the UPR or causing ER stress are genetic risk factors of disease as indicated

well experimental colitis induced by DSS exhibited a gene dosage effect with $Xbp1^{+/-(IEC)}$ mice demonstrating a phenotype intermediate between $Xbp1^{+/+(IEC)}$ and $Xbp1^{-/-(IEC)}$ mice [18]. Overactivation of IRE1 might play an important role in the development of this phenotype as a consequence of hypomorphic XBP1 function, as even deletion of one *Xbp1* allele was sufficient to cause massive splicing of *Xbp1* mRNA. IRE1α recruits several adaptor proteins and receptors, such as TRAF2 (TNF receptor associated factor 2), ASK1 (apoptosis signal-regulating kinase 1, encoded by *Map3k5*), BAX (BCL2-associated X protein), BAK (BCL2-antagonist/killer 1), TNFR1 (tumor necrosis factor receptor type 1, encoded by *Tnfrsf1a*), to form an UPRsome which can connect the UPR to inflammatory and apoptotic signaling [14, 21–24]. It might similarly be speculated therefore that genetic deletion of IRE1β as alluded to in the previous paragraph might result in insufficient UPR induction, which as a consequence may result in a similar overactivation of the remaining IRE1 isoform, IRE1α. This in turn would also be reflected in increased grp78 expression as observed in $Ire1\beta^{-/-}$ mice.

However, IRE1β also exhibits biological functions that appear to be independent of ER stress. Specifically, IRE1β negatively regulates chylomicron secretion in the intestinal epithelium [25]. Chylomicrons are large vesicles that are formed in the ER from intestinally absorbed fat and fat-soluble vitamins and secreted by the intestinal epithelium. Apolipoprotein B (apoB) lipidation by microsomal triglyceride transfer protein (MTP), an ER-resident chaperone, represents the rate-limiting step in chylomicron biosynthesis [26]. High-cholesterol and high-fat diets decrease Ire1β mRNA in wild-type mice, and $Ire1\beta^{-/-}$ mice that were fed such a diet secreted more chylomicrons from their IECs and developed more severe hyperlipidemia. This was due to IRE1β-, but not IRE1α-dependent post-transcriptional decay of MTP mRNA [25]. Interestingly, while IRE1β is not expressed in the liver where apoB-containing VLDL (very low density lipoprotein) particles are secreted in an MTP-dependent fashion, IRE1α exerts a similar profound effect on lipid metabolism in the liver [27–30]. The regulation of MTP function by IRE1β in IECs is also interesting from the perspective of the inflammatory phenotype observed in $Xbp1^{-/-(IEC)}$ mice, since MTP is co-opted by the immune system to transfer antigenic lipid onto the nascent grove of CD1d [31–33] (and other CD1 molecules [34]). CD1d is expressed on a variety of cells including IECs and dendritic cells (DCs) [35], and CD1d-restricted antigen presentation plays a major role in oxazolone colitis, a hapten-induced model of human ulcerative colitis that is mediated by CD1d-restricted, invariant natural killer T cells [31, 36]. Indeed, a small IEC line with decreased XBP1 expression through RNA interference exhibits increased CD1d-restricted antigen

Table 1 ER stress-related genes, their function and disease phenotype in vivo

ER stress-related genes	Genetic association	Function	Model	Phenotype
XBP1 (XBP1)	CD and UC (candidate gene study, deep sequencing) [18]	Major UPR transcription factor activated by IRE1	*Xbp1*$^{\Delta IEC}$ mouse (IEC-specific gene deletion)	ER stress; spontaneous enteritis; loss of mature Paneth cells and impaired bacterial handling; inflammatory hyperreactivity of the epithelium; reduction in goblet cells; increased sensitivity to experimental colitis (DSS)[18]
ORMDL3 (ORMDL3)	CD, UC, asthma, T1DM (genome-wide association studies)[81–85]	ER-resident transmembrane molecule; exact role in IEC not clear [86]	N/A	N/A
AGR2 (AGR2)	CD and UC (candidate gene study) [80]	Protein disulfide isomerase (PDI), disulfide bond formation in proteins within the ER, required for MUC2 production [40]	*Agr2*$^{-/-}$ mouse (expression in secretory IEC's, [37–39]	ER stress: increased grp78 expression and Xbp1 splicing; spontaneous granulomatous ileocolitis; alterations in Paneth and goblet cells [19, 38]
ERN2 (IRE1β)	Not reported	Splicing of Xbp1, regulated IRE1-dependent decay	*Ire1*β$^{-/-}$ mouse (expression specifically in intestinal and respiratory epithelium)	ER stress: increased grp78 expression; increased sensitivity to experimental colitis (DSS) [13]; increased intestinal chylomicron secretion [25]
ATF6 (ATF6α)	Not reported	Orchestrates UPR downstream of ATF6 when cleaved by S1P and S2P	*Atf6*α$^{-/-}$ mouse	Increased expression of ER stress genes and sensitivity to experimental colitis (DSS) [47]
DNAJC3 (P58^{IPK})	Not reported	ER-resident chaperone facilitating protein folding [48, 49]	*P58*$^{IPK-/-}$ mouse	Increased grp78 and CHOP expression and IRE1 phosphorylation; increased sensitivity to experimental colitis (DSS) [47]
MBTPS1 (S1P)	Not reported	S1P cleaves ATF6 and activates other bZIP transcription factors [50, 51]	S1P missense mutation by ENU mutagenesis (*woodrat* mouse)	Reduced grp78 and grp94 expression in DSS-induced colitis albeit increased susceptibility [50]
DDIT3 (CHOP)	Not reported	Downstream of PERK/ATF4, connects unresolved ER stress with apoptosis [14]	*Chop*$^{-/-}$ mouse	Protected from DSS and TNBS induced colitis [52]
MUC2* (MUC2)	Not reported (poor GWAS coverage due to gene structure)	Protein of the mucin family forming the intestinal mucus barrier	Missense mutation in *Muc2* (*Winnie* and *Eeyore* mouse)	MUC2 precursor accumulation accompanied by ER stress; spontaneous colitis [59, 60]
MUC19* (MUC19)	CD and UC (genome-wide association studies, deep sequencing) [81, 107]	Mucin component expressed in salivary glands and trachea, role in other compartments poorly understood [108]	N/A	N/A
HLA-B27* (HLA-B27)	Spondyloarthritis (genome wide association and linkage studies) [61]	HLA class I T-cell antigen presentation	Human HLA-B27/β_2-microglobulin transgenic rats	HLA-B27 prone to misfolding; UPR activation in myeloid cells; spondyloarthritis, ileitis, arthritis, inflammatory lesions of the urogenital tract, skin nail and heart [62]

*Not primarily involved in endoplasmic reticulum maintenance

presentation in vitro suggesting increased MTP and/or CD1d function when XBP1 function is hypomorphic [18].

Anterior gradient 2 (AGR2) in IEC homeostasis

AGR2 is an ER-resident protein disulfide isomerase (PDI) originally discovered in a screen for mRNAs selectively expressed in intestinal goblet cells [37]. It is also expressed in other secretory IECs such as Paneth cells and enteroendocrine cells, and also in $MSI1^{+}$ (Musashi-1) intestinal progenitor cells, with the highest expression noted in the ileum and colon [38, 39]. PDIs are a family of at least 19 thioredoxin-like domain-containing proteins critical for formation of correctly arranged disulfide bonds in proteins within the ER [40]. As already alluded to above, goblet cells secrete abundant quantities of mucus into the intestinal lumen that consists primarily of MUC2 which forms a net-like polymer [6, 41]. The large protein size of MUC2 (>5,000 aa) is a challenge for the ER and the Golgi apparatus of goblet cells and also Paneth cells, where it is processed as it involves extensive *O*-glycosylation of central mucin repeats and intra- and inter-chain disulfide bond formation in the cysteine-rich *N*- and *C*-terminal domains [6, 42]. Glycosylated, folded and multimerized mucins then enter secretory granules, from which they are released in the lumen. AGR2 is critically required for MUC2 mucin production in vivo, and in its absence, the intestinal epithelium exhibits profoundly increased expression of ER stress markers such as grp78 and *Xbp1* splicing, together with increased expression of pro-inflammatory cytokines [19, 38]. The increased production of inflammatory mediators in the context of unabated ER stress is a common theme in the intestinal epithelium as also observed in $Xbp1^{-/-(IEC)}$ mice (see above). Consistent with this, silencing of *Agr2* expression in a pancreatic cancer cell line increased ER stress induced by tunicamycin [19]. Importantly, $Agr2^{-/-}$ mice spontaneously develop severe ileocolitis characterized by multinucleated giant cells reminiscent of the granulomatous inflammation observed in human Crohn's disease (CD) [19]. Spontaneous ileocolitis was preceded by marked expansion in the Paneth cell compartment located at the bottom of small intestinal crypts, and mice with a germ-line, but not inducible, deletion of *Agr2* also were noted to exhibit abnormally positioned Paneth cells in the villi of the small intestine [19]. Interestingly, an independently generated $Agr2^{-/-}$ mouse model did not develop spontaneous ileocolitis, despite exhibiting increased colonic expression of pro-inflammatory cytokines and rectal prolapse at very old age [38]. A different gene targeting strategy (exons 2–4 in [19] and exons 2–3 in [38]) and different genetic backgrounds (although both ES cell lines were derived from inbred 129 mice) could account for this difference. However, another intriguing possibility is that differences in the intestinal microbiota could have been the precipitator of the specific phenotypic manifestations in each of these colonies. This notion may be particularly interesting, since the ileocolitic mice were held under conventional conditions, whereas those that did not develop spontaneous disease were maintained in a specific pathogen-free (SPF) environment [19, 38]. Further characterization of these models and their intestinal microbiota in different environments might therefore be highly informative, and could provide an intriguing paradigm of gene—microbiota interaction and their relation to ER stress.

ATF6 signaling and intestinal homeostasis

The active form of ATF6, generated through S1P and S2P at the Golgi membrane subsequent to sensing misfolded proteins in the ER, is a potent transactivator of UPR target genes, including grp78 (78kDa glucose-regulated protein; also known as BiP [Binding immunoglobulin Protein] and encoded by *Haspa5*), glucose-regulated protein 94 (Grp94) and $P58^{IPK}$ (protein kinase inhibitor of 58 kDa; encoded by *Dnajc3*) [12, 14, 43–45]. ATF6β (encoded by *Atf6b*) is distantly related to ATF6α (encoded by *Atf6*), albeit both isoforms are ubiquitously expressed. While $Atf6\alpha^{-/-}$ and $Atf6\beta^{-/-}$ single knock-out mice are viable, though sensitive to ER stress, double-knockouts are embryonically lethal, indicating functional redundancy during embryonic development [46]. Although wild-type bone marrow-reconstituted $Atf6\alpha^{-/-}$ mice (to allow studying the role of ATF6α in non-hematopoietic cells, hence in inference primarily in colonic epithelium) do not develop spontaneous intestinal inflammation, they are exquisitely sensitive to DSS colitis [47]. During the course of DSS colitis in wild-type mice, colonic IECs develop ER stress, reflected by increased expression grp78, ATF4, CHOP, and spliced XBP1, coinciding with the development of colitis [47]. This ER stress response was impaired in $Atf6\alpha^{-/-}$ mice reconstituted with wild-type bone marrow, and these mice exhibited decreased expression of the ER chaperones grp78, grp94, and $P58^{IPK}$ compared to bone-marrow reconstituted $Atf6\alpha^{+/-}$ mice, which also resulted in increased apoptosis in the colonic epithelium [47]. The impaired upregulation of $P58^{IPK}$, an ER-resident chaperone belonging to the DNAJ chaperone family which is involved in proper protein folding [48, 49], in and of itself is of particular relevance for the development of the increased severity of DSS colitis observed. $P58^{IPK-/-}$ mice reconstituted with wild-type bone marrow develop more severe mucosal damage, loss of goblet cells, and denser inflammatory infiltration compared to heterozygous littermate controls upon exposure to DSS [47]. $P58^{IPK}$ deletion results in a stressed ER in colonic IECs, characterized by increased expression of grp78 and increased phosphorylation of IRE1α, together with induction of pro-apoptotic CHOP [47].

Further evidence points towards the contribution of this branch of the UPR in mediating susceptibility to experimental colitis. Using a forward-genetic approach with *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis, missense mutations in S1P (*Mbtps1*, membrane-bound transcription factor peptidase site 1) in a mouse model termed *woodrat* have been discovered that increase the susceptibility to DSS colitis [50]. These mice exhibited diminished levels of colonic grp78 and grp94 expression upon DSS administration [50]. Experiments with bone-marrow chimeric mice pointed to non-hematopoietic cells as the origin of the *woodrat* phenotype, hence inferring a role of intestinal epithelial cells. In addition to ATF6α and β, S1P is also involved in the activation of other bZIP transcription factors, such as CREBH (CRE-binding protein H), OASIS (old astrocyte specifically induced substance), lZIP (leucine zipper protein), BBF2H7 (box B-binding factor 2 human homolog on chromosome 7), and CREB4 (CRE-binding protein 4) [50, 51]. These could also contribute to the colitic phenotype observed in the *woodrat* mouse model, albeit the overlap in ER stress chaperone regulation in *woodrat* and *Atf6α*$^{-/-}$ mice makes an ATF6-dependent mechanism involving the UPR most likely as other S1P-activated transcription factors are less closely mechanistically linked to the UPR.

The PERK/eIF2α/ATF4/CHOP branch of the UPR and gastrointestinal disease

As already alluded to above, the CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) is induced in the colitis observed in XBP1-, ATF6α-, and P58^{IPK}-deficient mice, indicative of activation of the PERK/eIF2α branch of the UPR due to ER stress. CHOP has an important role in inducing apoptosis in cells that exhibit unabated ER stress [14]. Indeed, *Chop*$^{-/-}$ mice are protected from DSS colitis, which is associated with a decrease of apoptotic cells observed during colitis [52]. *Chop*$^{-/-}$ mice were similarly protected from colitis induced by trinitrobenzene sulfonic acid (TNBS) [52], a hapten-induced colitis model [53]. In light of the importance of IL-23R signaling for the evolution of intestinal inflammation in IBD [54–57], it is notable that CHOP markedly enhances TLR-induced IL-23 production in myeloid cells via increased binding of CHOP to the IL-23 p19 (*Il23A*) promoter under conditions of ER stress [58].

Missense mutations in Muc2 and spontaneous colitis

An ENU-induced forward-genetic screen has revealed two notable mouse models with spontaneous colitis resembling human UC, which are caused by two independent missense mutations in the *Muc2* gene [59]. These mutant mice (*Winnie* and *Eeyore*) exhibit evidence of altered MUC2 biosynthesis with accumulation of MUC2 precursor, and a decrease in stored, properly glycosylated mucin in goblet cell granules. This was accompanied by ultrastructural and biochemical (grp78, XBP1 splicing) evidence of ER stress [59]. The causative missense mutations locate to the MUC2 oligomerization domain, and in vitro transfection studies of normal and variant oligomerization domains indicated that the associated misfolding caused ER stress. This is a very interesting model of spontaneous pathology, albeit it is inherently complex to tease out the relative contribution of ER stress in goblet cells, and the contribution of a diminished mucus layer to disease pathology, since *Muc2*$^{-/-}$ mice also develop spontaneous colitis [60].

HLA-B27 and the UPR

Individuals with the human class I major histocompatibility allele HLA-B27 are at significant risk for developing spondyloarthritis, a systemic inflammatory condition not only involving joints, but also inflammation of the small intestine among other immune phenomena [61]. HLA-B27/human β_2-microglobulin transgenic rats recapitulate the human disease as they develop spondyloarthritis and peripheral arthritis, ileitis, and further inflammatory lesions in the genital tract, skin, nails, and heart [62]. Classical MHC I-restricted CD8αβ$^+$ T cells do not appear to be required for pathogenesis as their depletion has no effect on arthritis or gastrointestinal inflammation in HLA-B27/β_2m transgenic rats [63]. Notably, the UPR is activated in macrophages from HLA-B27/β_2m transgenic rats, implying that HLA-B27 misfolding, which HLA-B27 is inherently prone to, induces ER stress [64]. Induction of the UPR in myeloid cells from HLA B27/β_2m-transgenic rats results interestingly in increased IL-23 expression upon stimulation with the TLR4 ligand LPS, and indeed IL-23 expression is increased in colonic tissue of transgenic rats concurrently with the development of intestinal inflammation [65]. This was associated with increased IL-17 expression, a downstream target of IL-23, in CD4$^+$ T cells [65]. Altogether, these data suggest a possible link of HLA-B27 with intestinal inflammation on the basis of increased IL-23 expression due to an activated UPR consequent to HLA-B27 misfolding. Whether this is CHOP-mediated remains to be defined [58].

ER stress and activation of the UPR in human gastrointestinal tissue

Evidence of unresolved ER stress and an activated UPR in IECs has been reported in both forms of IBD, Crohn's disease (CD) and ulcerative colitis (UC) [18, 59, 66–68], obesity [69], HIV/AIDS [70], and during the course of injury associated with ischemia/reperfusion [71].

With regard to IBD, evidence of ER stress has been reported in IECs isolated from both inflamed and non-

inflamed regions of the small and large intestine in patients with CD and UC [18, 59, 66–68]. In CD, increased grp78 expression and *XBP1* splicing was noted in intestinal biopsies [18, 68] and grp78 localized to small intestinal and colonic IECs on immunohistochemistry [68]. In the latter study, ER stress in IECs was associated with downregulation of the xenobiotic transporter ATP-binding cassette G2 (ABCG2) from the cell surface [68]. Another in-depth study has analyzed IECs from non-inflamed tissues of patients with UC [67]. In this context, colonic IECs exhibited substantially increased *XBP1* splicing, indicative of increased IRE1 activity, together with increased expression of Grp78, Grp94, and EDEM1 (ER degradation enhancer, mannosidase alpha-like 1) and increased cleavage of ATF6α to its transcriptionally active p50 form compared to IECs obtained from healthy control patients [67]. These findings were accompanied by an expansion of the ER surface area as revealed through transmission electron microscopic studies [67]. Altogether, these results indicate massive ER stress in the UC epithelium, even in the non-inflamed state. Considering that only a small fraction of IBD patients are predicted to harbor risk-conferring polymorphisms in UPR and ER stress-related genes [18, 56, 72], it is remarkable that all non-inflamed UC biopsies studied exhibited ER stress, while none of the control subjects did [67], implying unresolved ER stress as a general feature of the UC epithelium. Notably, while the aforementioned data indicate activation of the IRE1/XBP1 and ATF6 branches of the UPR, phosphorylation of eIF2α was observed to be decreased in UC epithelium compared to healthy controls [67]. This was accompanied by decreased ATF4 and CHOP, and increased GADD34 (growth arrest and DNA damage-inducible protein 34, encoded by *Ppp1r15a*) expression in UC compared to healthy controls, implying a selective hypomorphic activation of the eIF2α/ATF4/CHOP branch of the UPR in UC [67]. However, circumstantial evidence presented in this paper suggested that hypomorphic eIF2α activation was not necessarily a consequence of decreased PERK activation [67]. The suspected consequent deregulation of protein translation initiation was reflected in alterations in mRNAs actively translated from polysomes [67]. The mechanistic basis and functional importance of this selective hypomorphic eIF2α activation in the context of overt ER stress in the UC epithelium is currently unclear.

Of further note in this context is also the regulation of the chaperone grp96, one of the most abundant ER proteins, which is specifically involved in the folding, assembly, and export of specific proteins in the ER, including Toll-like receptors (TLRs) and as such involved in innate immunity toward pathogens [73]. The ER stressed intestinal epithelium of the ileum in CD exhibits strong expression of grp96 at the apical plasma membrane together with CEACAM6 (carcinoembryonic antigen-related cell adhesion molecule 6), where it is essential for the invasion of adherent-invasive *Escherichia coli* (AIEC) [74]. AIEC can adhere and invade the intestinal epithelium, and are preferentially found in patients with CD, where they colonize the ileal mucosa [75]. Thus, ER stress may result in an intestinal epithelium that is more prone to binding pro-inflammatory pathobionts such as AIEC which serve to further promote the activity of the disease.

Apart from IBD, an activated UPR in IECs, in particular Paneth cells, has been demonstrated in obesity [69]. The authors were initially prompted to investigate Paneth cells for their antimicrobial function because of some notable similarities in the alterations found in the intestinal microbiota (i.e., increase in *Firmicutes* over *Bacteroidetes*) in obesity and IBD compared to healthy, normal weight individuals. Interestingly, Paneth cells of severely obese subjects contained decreased levels of human α-defensin 5 (HD5, encoded by *DEFA5*) and lysozyme protein compared to normal weight subjects, despite unaltered mRNA expression of both transcripts [69], implying a translational block. Indeed, Paneth cells from severely obese patients exhibited ultrastructural and biochemical evidence of ER stress, reflected by increased grp78 and ATF4 expression compared to normal weight subjects [69]. Furthermore, grp78 expression in Paneth cells was inversely correlated with lysozyme protein expression [69]. These observations fit very well with the long-known activation of the UPR and unresolved ER stress in the obese liver, which regulates insulin sensitivity [76].

Finally, UPR induction has also been implicated in the mechanisms that lead to intestinal leakiness during the course of ischemia/reperfusion injury in the intestine. In an interesting experimental system of jejunal ischemia/reperfusion injury in patients undergoing pancreaticoduodenectomy, ER stress in Paneth cells was observed as deduced from evidence for increased expression of grp78, CHOP, and GADD34, and increased splicing of *XBP1* mRNA [71]. This was associated with increased apoptosis of Paneth cells. Recapitulating this model system in Sprague–Dawley rats, ER stress-associated Paneth cell loss, or cytotoxic depletion of Paneth cells through administration of dithizone, which complexes with zinc found in high concentrations in their granules [77], were associated with increased bacterial translocation and elevated circulating levels of TNFα and IL-6 [71]. These data suggest that ER stress-associated Paneth cell loss may be an important contributor to the leakiness of the intestine after ischemia/reperfusion injury. ER stress might also contribute to the leakiness of the intestine in human immunodeficiency virus (HIV) infection and the acquired immunodeficiency syndrome (AIDS). Specifically, duodenal biopsies from patients with HIV and AIDS display increased levels of XBP1s and CHOP expression, along with increased IL-1β, CD3ε, and HLA-DR expression, and IECs exposed to HIV-1 Vpr results in a similar UPR activation together with pro-inflammatory gene expression [70]. It might be speculated that ER stress mechanisms

might contribute to the leakiness of the intestine in AIDS patients, which exhibit a pattern of serological markers of bacterial translocation very similar to that described in CD [78]. Of additional note, specific HIV protease inhibitors used in the treatment of HIV can induce ER stress in the intestinal epithelium and thereby might also disrupt barrier integrity [79].

Genetic evidence for involvement of ER stress-related mechanisms in human gastrointestinal tissue

Genetic association studies have revealed several genetic loci that are significantly associated with risk for CD and UC (Fig. 1, Table 1). A candidate gene study prompted by the spontaneous enteritis arising in *Xbp1*$^{-/-(IEC)}$ mice that surveyed the *XBP1* locus and its wider vicinity revealed an association signal for this locus for IBD, which was replicated in two further cohorts of patients and thereby confirmed the association with CD and UC [18]. The apparent lack of linkage disequilibrium at the larger *XBP1* locus suggested that the locus association signal might be based on individual rare variants. A deep sequencing experiment of the coding region of *XBP1* and its promoter revealed an approximately threefold larger number of rare single nucleotide polymorphisms (SNP) at this locus in IBD patients compared to healthy controls [18]. Among those were non-synonymous (ns) SNPs which only occurred in IBD and not in healthy controls. Two of those were functionally characterized via transfection of wild-type and mutant expression vectors into IEC lines or *Xbp1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) [18]. These IBD-only variants resulted in decreased induction of UPR target genes, while a further nsSNP variant that occurred at similar frequency in healthy controls and IBD patients was indistinguishable from the wild-type variant in inducing UPR target genes [18]. Altogether, these data indicated that the *XBP1* locus was an IBD risk locus, with rare variants constituting the basis for this association signal. The hypomorphic character of these rare variants fit mechanistic insights gleaned from mice with hypomorphic *Xbp1* function in IECs, which develop spontaneous ileitis/enteritis [18]. Furthermore, the mouse model showed that deletion of one *Xbp1* allele in IECs in vivo is sufficient to cause spontaneous enteritis as well as profound induction of ER stress as revealed through the massive hyperactication of IRE1 endoribonuclease activity [18]. This suggests that even minor reductions in XBP1 function might have major effects on IEC function through this mechanism.

A further genetic association that was revealed through a candidate gene study is *AGR2*, which was also found to be associated with CD and UC [80]. As noted above, *Agr2*$^{-/-}$ mice exhibit ER stress in their intestinal epithelium and develop ileocolitis with characteristic granuloma-like features.

Finally, an association between Orosomucoid-like 3 (*ORMDL3*) and the risk for developing CD [81] and UC [82] has been uncovered through unbiased genome-wide association studies (GWAS). Interestingly, *ORMDL3* is also associated with asthma and type 1 diabetes mellitus [83–85]. ORMDL3 is an ER-resident transmembrane molecule primarily expressed in epithelial cells [86], which affects the UPR, although its exact role is not yet entirely clear. Transfection of ORMDL3 into lung epithelial cells selectively activated ATF6α, which consequently transactivated SERCA2b (sarcoplasmic/endoplasmic reticulum calcium ATPase 2, encoded by *Atp2a2*) transcription, which is important in airway remodeling [86]. In this model system, the other branches of the UPR were not activated by ORMDL3 over-expression. In bronchial epithelial cells, ORMDL3 expression was substantially increased by Th2 cytokines in a STAT6-dependent manner [86]. A further study in human embryonic kidney cell line HEK293T suggested that ORMDL3 binds and inhibits the sarco-endoplasmic reticulum Ca^{2+} pump (SERCA), which results in reduced ER Ca^{2+} concentrations and an increased UPR manifested by increased phosphorylation of eIF2α [87]. Increased IRE1 activity was not present as *XBP1* mRNA splicing remained unaltered upon overexpression of ORMDL3 [87]. In contrast to these observations, another study reported that overexpression of ORMDL3 in HEK293T cells decreased basal and tunicamycin-induced UPR, whereas ORMDL3 silencing resulted in an exaggerated UPR after tunicamycin stimulation as deduced from binding assays to a *cis*-acting UPR element (UPRE) reporter specific for ATF6 [82, 88]. The biological function of ORMDL3 in the ER is complex, as it also acts as a negative regulator of sphingolipid production via formation of a complex with serine palmitoyltransferase, the rate-limiting step in sphingolipid biosynthesis [89]. The exact role of ORMDL3 in IBD clearly needs further study in relevant model systems.

In concluding this discussion of genetic risk factors in IBD that are associated with factors involved in the UPR, it is important to mention HLA-B27. Specific HLA-B27 subtypes are associated with risk for developing spondyloarthropathies, which are often associated with mucosal inflammation in the ileum [90].

Points of therapeutic intervention

Considering that IECs appear particularly susceptible to ER stress, and impairment in the UPR in IECs can lead to intestinal inflammation, therapeutic intervention appears as an attractive strategy. Indeed, small molecule chaperones, such as tauro-ursodeoxycholic acid (TUDCA) and 4-phenyl butyrate (PBA), have been reported that contribute to proper protein folding in the ER and thus alleviate ER stress. The

study of such compounds has been pioneered in the field of obesity and insulin resistance [91, 92], and aspects related to therapeutic manipulation of ER stress are extensively discussed in Ozcan and colleagues in this review series [93].

The utility of TUDCA and PBA has also been investigated in models of intestinal inflammation. TUDCA and PBA have been demonstrated to alleviate ER stress in the colonic epithelium in DSS colitis, which was associated with amelioration of intestinal inflammation in the colon [47]. Treatment with either compound also alleviated the more severe course of DSS colitis observed in $Atf6\alpha^{-/-}$ and $P58^{IPK-/-}$ mice reconstituted with wild-type bone marrow, implying indeed an ER stress-mediated mechanism underlying the more severe disease course and specifically an important role for ER stress and therapeutic targeting of the intestinal epithelium [47]. Finally, in $Il10^{-/-}$ mice that received piroxicam for the induction of colitis, TUDCA, or PBA treatment similarly ameliorated experimental disease [47]. Altogether, these data indicate that small molecular chaperones may be efficient in alleviating ER stress, thereby decreasing experimentally induced intestinal inflammation. Based on the notion that IECs are particularly important for ER stress-associated intestinal inflammation, topical treatment at the inner body surface via local delivery may represent an attractive treatment paradigm to minimize systemic effects of these drugs.

In this context, it is noteworthy that the ileal epithelium is naturally exposed to TUDCA, a tertiary bile salt [94]. TUDCA is a taurine conjugate of UDCA, though the latter appears to be substantially more potent in alleviating ER stress in IECs [94]. In contrast to UDCA, which is substantially more lipophilic and thereby can seamlessly traverse biological membranes including the ER, TUDCA requires active cellular uptake via the ileal bile acid:sodium symporter [94]. UDCA is currently in common clinical use in cholestatic liver diseases, primary biliary cirrhosis and primary sclerosing cholangitis, and it might be speculated that part of the long-known efficacy of this compound might be related to alleviation of ER stress. However, bile acids have multiple other biological functions independent of ER stress alleviation, which could also contribute to their clinical efficacy [95].

Concluding remarks

The UPR is a critical component of IEC homeostasis in the intestine, and impairment can lead to the original initiation of intestinal inflammation as observed in IBD (Fig. 1, Table 1). While genetic impairment of UPR-related mechanisms has been well documented, our understanding of environmental, microbial, and dietary factors that impact on UPR function in the intestine is only beginning to develop. Specific bacterial toxins can activate (e.g., Shiga toxin subAB proteolytically cleaves grp78 and thereby activates all three branches of the UPR [96–98]) and inhibit (e.g., trierixin, which inhibits XBP1 splicing [99, 100]) the UPR. Given this, it is highly likely that many more bacterial metabolites might affect UPR function and remain to be elucidated. One example might be the microbial metabolism of bile acids such as TUDCA and UDCA associated with microbial dysbiosis in IBD [101], which might determine the availability of endogenous "ER stress relievers" at the mucosal surface. Dietary intake might also have an important influence on the IECs capacity to withstand ER stress with the notion that there are dietary agents which enhance or inhibit UPR function and thus are either beneficial or deleterious to the intestinal epithelium, respectively. One example is glutamine, which has been shown to alleviate ER stress in the epithelium and decrease the severity of TNBS colitis in rats [102]. Specifically, glutamine decreased ATF6, ATF4, and CHOP expression, along with decreased IRE1 phosphorylation and XBP1 splicing, which was associated with decreased induction of apoptosis in IECs [102]. A further example is related to the oral intake of iron [103]. In the $Tnf^{\Delta ARE}$ model of spontaneous ileitis, changing from a regular murine chow diet to an iron sulfate-free diet protected from intestinal inflammation, which was associated with alleviation of ER stress and pro-apoptotic mechanisms in IECs [103]. Interestingly, ER stress in the IEC line MODE-K increased their susceptibility to the cytotoxic effector function of T cells from $Tnf^{\Delta ARE}$ mice. Since the change in diet had a profound impact on the composition of the intestinal microbiota [103], the exact cause–effect relationship of the beneficial effects of an iron sulfate-free diet with regard to ER stress, direct anti-inflammatory mechanisms, and microbial effects are hard to discern. Nonetheless, these examples highlight the variety and substantial importance of non-host factors that impact on the UPR in IECs in the intestine. These "non-host" factors that directly or indirectly impact on the UPR could potentially constitute the mechanistic basis for why ER stress appears to be a very common and typical feature of IBD IECs even in the non-inflamed state [18], in particular in UC [67].

An innate immune response is per se an important, and evolutionary conserved, inducer of ER stress, as elegantly demonstrated in *Caenorhabditis elegans* [104]. Considering the massive induction of protein synthesis associated with inflammatory mediator secretion as a consequence of innate immune activation of cells, which may arise from a bona fide microbial signal or as part of an early innate immune signal of the host (e.g., pro-inflammatory cytokines), such a requirement for an efficient UPR may appear unsurprising indeed [105]. Consistent with this, in IECs pro-inflammatory cytokines such as TNFα, induce, and anti-inflammatory cytokines such as IL-10 inhibit ER stress [66]. This requirement for an efficient UPR as an integral part of an innate immune mechanism might not only be relevant for IECs, but appears also to

be relevant for CD8$\alpha\beta^+$ intraepithelial lymphocytes (IELs), which exhibit attenuated granzyme B-dependent cytotoxicity when grp78 is rendered hypomorphic [106]. For these reasons, ER stress-related mechanisms in IECs (and potentially IELs and other immune cells) may therefore also play an important role in perpetuating intestinal inflammation arising from a variety of different pathophysiological pathways and as a corollary be associated with the development of colorectal neoplasia, a major sequelae of these diseases. In conclusion, the UPR plays a fundamentally important role in the intestine, in particular within the intestinal epithelium, where it integrates host, microbial, and environmental signals, and contributes to the pathophysiology of IBD.

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