ENDOPLASMIC RETICULUM STRESS IN INTESTINAL EPITHELIAL CELLS AND INFLAMMATORY BOWEL DISEASE

By

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To my wife and parents

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PREFACE

Chapter I is an introduction to the unfolded protein response and a review of our current understanding of endoplasmic reticulum stress and the UPR in inflammatory bowel disease (some contents from two publications of Cao *et al*: *Curr Biol. 2012 Aug 21;22(16):R622-6.*; *Expert Opin Ther Targets. 2013 Apr;17(4):437-48. doi: 10.1517/14728222.2013.756471. Epub 2013 Jan 17.*). Chapter II is a publication by Cao *et al* (*Inflamm Bowel Dis. 2012 Sep;18(9):1735-42.*). Chapter III is another publication by Cao *et al* (*Gastroenterology. 2013 May;144(5):989-1000.e6. doi: 10.1053/j.gastro.2013.01.023. Epub 2013 Jan 18.*). Chapter IV presents data on the role of eIF2α phosphorylation in intestinal epithelial function and is being prepared for submission by Cao *et al.* Chapter V summarizes the findings during my Ph.D. research, current projects, and my perspective for the future directions of the ER stress-IBD field (one figure from *Expert Opin Ther Targets. 2013 Apr;17(4):437-48. doi: 10.1517/14728222.2013.756471. Epub 2013 Jan 17.*).

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CHAPTER I

INTRODUCTION

In eukaryotic cells, the endoplasmic reticulum (ER) is a membrane-bound organelle that is crucial for the folding and maturation of proteins, lipid biosynthesis, and homeostasis of intracellular Ca^{2+} and reduction-oxidation (redox) potential. Protein folding and modification in the ER is highly sensitive to disturbances of ER homeostasis, including altered glycosylation, ER Ca^{2+} depletion, increased mRNA translation, oxidative stress, energy deprivation, metabolic challenge, and inflammatory stimuli. The accumulation of unfolded and misfolded proteins in the ER lumen, termed ER stress, activates intracellular signaling pathways to resolve the protein folding defect. This unfolded protein response (UPR) increases the capacity of ER protein folding and modification, reduces global protein synthesis, and activates ER-associated protein degradation (ERAD). If ER stress is too severe or chronic, or the UPR is compromised and not able to restore the protein-folding homeostasis, numerous apoptotic signaling pathways are activated (Figure 1-1) [1-3]. Pre-clinical and clinical studies in the last decade indicate that ER stress and the UPR have a significant impact on the pathogenesis of multiple human diseases including metabolic disease, neurodegenerative disease, inflammatory disease, and neoplastic disease (Figure 1-2) [3-5]. With our increased

understanding of ER protein-folding processes and the mechanistic roles of UPR pathways in adaptive and apoptotic responses in various cells and tissues, it is now possible to develop novel therapeutics to improve ER protein-folding and to target specific UPR pathways to resolve protein folding defects and restore ER homeostasis in human pathologies.

I. The unfolded protein response

In mammalian cells, three protein sensors on the ER membrane initiate UPR signaling: inositol-requiring kinase 1 (IRE1), pancreatic ER eIF2α kinase (PERK), and activating transcription factor 6 (ATF6). Binding of the ER luminal protein chaperone BiP/GRP78 to the UPR sensors prevents their signaling. Based on the competition-binding model, unfolded and/or misfolded proteins in the ER lumen compete with the ER stress sensors for binding to BiP. Upon accumulation of unfolded/misfolded proteins, the transmembrane sensors are activated due to the release of BiP from their luminal domains [2, 3].

The most conserved UPR branch is mediated by IRE1, a type I transmembrane protein with both an endoribonuclease (RNase) domain and a Ser/Thr kinase domain in its cytosolic portion. In mammals there are two IRE1 genes, IRE1α and IRE1β, which differ in their expression patterns. Only IRE1α is expressed ubiquitously, while IRE1β expression is primarily restricted to the intestinal epithelium. Upon release from BiP, the luminal domain of IRE1α dimerizes in the plane of the ER membrane, leading to *trans*-autophosphorylation and activation of the kinase and RNase activities. Activated IRE1α removes a 26-base intron from an mRNA causing a translational frame-shift, which is

then translated to produce a potent CREB/ATF basic leucine zipper-containing (bZIP) transcription factor X-box-binding protein 1 (XBP1). XBP1 transactivates a number of genes that control ER protein folding, maturation, intracellular transport, ERAD, as well as phospholipid biosynthesis and membrane expansion. The IRE1-XBP1 pathway plays an important role in the differentiation, function, and survival of a variety of professional secretory cells in mammals. In addition to initiating splicing of *Xbp1* mRNA, IRE1 also degrades a subset of ER-localized mRNAs in a stress-dependent manner, which is called regulated IRE1-dependent decay of mRNA (RIDD). Recent studies also show that IRE1 can degrade microRNAs (miRs) leading to activation of apoptotic and inflammatory pathways [6, 7]. As a protein kinase, IRE1α also integrates the ER protein-folding status with pro-inflammatory responses through the physical interaction with TNFα receptor-associated factor 2 (TRAF2) and subsequent activation of the JNK and NF-κB pathways [2, 3, 8].

PERK is a type I transmembrane protein with a cytosolic Ser/Thr kinase domain. Upon activation, PERK phosphorylates Ser51 on the α subunit of eukaryotic translation initiation factor 2 (eIF2α), which in turn attenuates translation initiation to shut down global protein synthesis and reduce the ER protein-folding load. In mammals, three additional eIF2α kinases, dsRNA-activated protein kinase (PKR), general control nonrepressed 2 kinase (GCN2), and heme-regulated eIF2α kinase (HRI), phosphorylate eIF2α at Ser51 in response to different cellular stresses. In response to ER stress, eIF2α phosphorylation-mediated translation attenuation is transient and is reversed by GADD34 and CReP, two regulatory targeting subunits of protein phosphatase PPP1, which directly target eIF2α dephosphorylation. In addition to global translational

inhibition, phosphorylated eIF2α selectively promotes translation of a growing number of mRNAs, including activating transcription factor 4 (ATF4) mRNA encoding a bZIP transcription factor that activates genes for ER chaperones, transcription factors, as well as components of anti-oxidative responses, autophagy, and intracellular trafficking machinery. One downstream target of ATF4 is CCAAT/enhancer-binding protein homologous protein (CHOP/GADD153), a bZIP transcription factor that plays a crucial role in ER stress-induced apoptosis [3, 9].

In contrast to IRE1 and PERK, ATF6 is a type II transmembrane protein with a CREB/ATF bZIP domain at its N-terminus. In mammals there are two ATF6 genes, ATF6α and ATF6β, where only ATF6α signals UPR gene induction. Upon ER stress, ATF6 is freed from the ER chaperone BiP and then translocates to the Golgi apparatus, where it is sequentially cleaved by proteases S1P and S2P at the transmembrane site. The released ATF6α cytosolic fragment p50 migrates to the nucleus and functions as a potent transcription factor for numerous ER chaperone genes, including *BiP*, *Grp94*, and $P58^{IPK}$, and ERAD functions. ATF6α is essential for optimal protein folding, modification, and secretion in response to ER stress. In the absence of ATF6α, cells cannot survive chronic ER stress. In addition to ATF6, additional regulated intramembrane proteolysis (RIP)-regulated bZIP transcription factors exist in mammals, including CREBH, Luman, and OASIS, which show diverse biological functions in different cell types [3, 10].

II. ER stress, mitochondrial dysfunction, and apoptosis

UPR signaling is an important adaptive mechanism in response to ER protein folding defects. However, prolonged ER stress leads to activation of the pro-apoptotic

UPR, which is mainly mediated by the UPR components CHOP and IRE1α, as well as crosstalk between ER and mitochondria.

It is now clear that CHOP is a master regulator of ER stress-induced apoptosis as demonstrated by numerous *in vitro* and animals disease models [11]. Some studies have suggested that CHOP induces cell death through suppression of pro-survival protein Bcl-2 and induction of pro-apoptotic protein Bim. In addition, CHOP activates other pro-apoptotic factors including death receptor 5 (Dr5) and telomere repeat binding factor 3 (TRB3), which are important mediators of ER stress-induced apoptosis in multiple cancer cell lines. Other mechanisms implicated in CHOP-mediated cell death include oxidative stress and pro-apoptotic Ca^{2+} signaling. ER oxidase 1α (ERO1α) enhances disulfide bond formation and plays an important role in oxidative protein folding in the ER. During ER stress, ERO1α is transactivated by CHOP and may directly generate reactive oxygen species (ROS) in the ER. Additionally, induced ERO1α stimulates inositol-1,4,5-trisphosphate receptor (IP3R)-mediated Ca^{2+} release from the ER, which then activates Ca^{2+} sensing kinase CaMKII and its downstream pro-apoptotic pathways [1-3].

As the most conserved ER stress transducer, IRE1α is required for murine embryonic development and optimal UPR induction in some professional secretory cells. However, increasing evidence suggests that some non-secretory cells may survive chronic ER stress better in absence of IRE1α. First of all, IRE1α can contribute to pro-inflammatory and pro-apoptotic signaling in the cell through the activation of JNK. IRE1α physically interacts with Bax and Bak, two pro-apoptotic Bcl-2 family members that promote mitochondrial-dependent cell death. In addition, while RIDD may help

alleviate ER stress by suppressing ER-associated mRNA translation, it may induce apoptosis during prolonged ER stress [1, 2].

Mitochondrial apoptotic pathways also play a critical role in ER stress-induced cell death. During ER stress, Ca^{2+} released from the ER is taken up by nearby mitochondria, which leads to mitochondrial damage, production of ROS, and pro-apoptotic signaling. ER and mitochondria are physically and functionally connected by mitochondria-associated ER membranes (MAMs), which are vital for regulating Ca^{2+} uptake into the mitochondrial matrix [12]. The major Ca^{2+} channels including IP3R and the voltage-dependent anion channel (VDAC) are enriched in MAMs [13]. A recent study showed that the ER stress sensor PERK is also a MAM component, which functions to maintain the ER-mitochondria juxtaposition and promote mitochondrial stress and apoptosis upon oxidative stress [14]. Bax inhibitor-1 (BI-1), a modulator of IRE1 activation on ER membrane, controls mitochondrial bioenergetics, redox signaling, and autophagy by regulating IP3R-dependent Ca^{2+} efflux [15].

III. Implications of ER Stress and the UPR in intestinal epithelial cell function and inflammatory bowel disease

Inflammatory bowel diseases, including Crohn's disease and ulcerative colitis, are the most prevalent gastrointestinal disorders in the US. While ulcerative colitis usually only affects the large intestine, Crohn's disease can inflame any part of gastrointestinal tract, especially terminal ileum and colon. It is estimated that ~1.4 million Americans suffer from this disease, with more than 700,000 physician visits, 100,000 hospitalizations, and 119,000 disabilities every year, leading to a health care cost > $1.7

billion per year (http://www.cdc.gov/ibd/). In spite of dozens of years of studies including 163 genomic loci identified to associated with IBD (90 with Crohn's disease, 73 with ulcerative colitis) [16], the etiology of this debilitating disease remains elusive. There is currently no cure for the disease that commonly requires a lifetime of care for patients with this chronic condition. Development of therapies for IBD faces an extraordinary challenge due to our poor understanding of the disease. Additionally, current medications which target the patients' immune system exhibit significant risks and side effects [17].

The mammalian digestive tract is continuously exposed to a large number of antigens including trillions of microbes and various food metabolites. Intestinal homeostasis is achieved by sophisticated regulation of the immune response in the gut and peripheral tissues. Immune cell populations, including both innate and adaptive immune cells, play the central role in orchestrating the homeostasis-inflammation balance in gastrointestinal tract. Recently, increased evidence suggests that the epithelial cells lining the bowel wall actively participate in the maintenance of mucosal homeostasis instead of functioning as an inert barrier. Murine and human intestinal epithelia contain four secretory cell types: Paneth, goblet, enteroendocrine, and tuft cells, all of which are differentiated from a common, constantly renewing $Lgr5^+$ intestinal stem cell population [18, 19]. Paneth cells are pyramid-like columnar epithelial cells which reside in the bottom of crypts of the small intestine [20], and play a crucial role in innate immunity and host defense against bacteria, fungi and some viruses. Paneth cells directly sense intestinal bacteria and their products through cell-autonomous MyD88-dependent toll-like receptor activation, triggering expression and secretion of multiple antimicrobial factors including cryptdins/α-defensins, lysozyme, and phospholipase A2 [21]. Previous studies indicate that

Paneth cells are essential for limiting the penetration by commensal and pathogenic microorganisms across intestinal barrier, adjusting the balance among various bacterial populations, and maintaining intestinal homeostasis. The dysfunction of Paneth cells including reduced secretion of antimicrobial peptides has been linked to Crohn's ileitis [22]. Goblet cells are mucin-producing cells in both small and large intestines. They secrete a large amount of gel-forming and cell-surface mucins under normal physiological conditions. MUC2 mucin, the major component of the mucus layer in the bowel, undergoes N-glycosylation in the ER and O-glycosylation in the Golgi apparatus before being secreted into the intestinal lumen [23]. The intestinal mucus layer prevents the infiltration of bacteria, viruses, fungi, and their products into the mucosa and is critical for intestinal homeostasis. Impaired goblet cell function, e.g. genetic ablation of *Muc2*, led to spontaneous colitis and colitis-associated colon cancer in mice [24]. Goblet cell abnormality, e.g. decreased cell number and diminished mucin granules, is a hallmark of ulcerative colitis [25]. However, the physiological importance of goblet cell pathology in ulcerative colitis is not fully understood.

Recently, ER stress and the UPR have been linked to the pathogenesis of IBD [26]. Human genetic studies of IBD have identified primary genetic abnormalities in several genes including *XBP1*, *AGR2*, and *ORMDL3* that encode proteins associated with ER function [27]. Animal studies demonstrated that the induction of ER stress through tissue-specific deletion of the gene encoding XBP1 results in the absence of Paneth cells and spontaneous inflammation in small intestine, along with impaired host defense to bacterial infection. *Xbp1* conditional knockout mice also displayed reduced number and defective mucin secretion of goblet cells in the gut [28]. Interestingly, loss of *Xbp1* alleles

caused massive activation of IRE1α in ileal epithelium, which stimulated JNK and NFκB pathways and production of inflammatory mediators in the gut. Recently, the same group showed that treating the *Xbp1* conditional knockout mice with an inhibitor of IκBα phosphorylation (to block NF-κB pathway) was sufficient to prevent Paneth cell death and mitigate spontaneous ileitis in these mice [29]. However, how XBP1 regulates goblet cell function in the gut is still unclear. The deficiency of Agr2, which functions in ER protein folding as a protein disulfide isomerase, leads to ER stress in the intestinal epithelium, disruption of Paneth cell homeostasis, and granulomatous ileocolitis which phenocypied human Crohn's disease [30]. In another study, Agr2 was found to play a crucial role in the maturation and secretion of MUC2 mucin in murine colonic goblet cells [31]. *ORMDL3* is linked to ER Ca^{2+} homeostasis and activation of the UPR [32, 33]. However, its role in IBD remains to be elucidated.

In addition to the genes that have been associated with human IBD, studies of other UPR components also highlight the important role of ER stress in IEC function and intestinal homeostasis. IRE1β, an IRE1 isoform specifically expressed in mammalian gastrointestinal tract and airway mucous cells [34], was protective against chemical-induced colitis in mice with an unclear mechanism [35]. This is the first study that linked the UPR to IEC function and intestinal inflammation. Recently, IRE1β was shown to optimize the folding and secretion of MUC2 mucin in goblet cells by degrading *Muc2* mRNA. In mice deleted in *Ire1β*, misfolded MUC2 precursors accumulated in the ER lumen of colonic goblet cells, which exhibited distended ER and ER stress [36]. How IRE1β regulates goblet cell function and mucosal homeostasis during enterocolitis and infection in the gut remains to be elucidated. In contrast, ablation of *Chop* alleviates chemical-induced colitis

in mice [37]. However, it is not clear whether this phenotype is due to the loss of CHOP in epithelial or hematopoietic compartment since the authors used a whole-body deletion of *Chop* in that study. Another study showed that a hypomorphic mutation in the gene encoding site-1 protease (S1P) in mice increases the susceptibility to experimental colitis [38]. However, given S1P targets several ER stress-induced bZIP transcription factors including ATF6α/β, Luman, OASIS, CREBH, and SREBPs, it is possible that more than one of these transcription factors affects IEC function and mucosal homeostasis [3]. Recently, OASIS was shown to regulate the terminal differentiation of goblet cells; *Oasis*$^{-/-}$ mice exhibited abnormal ER and mucous vesicles in colonic goblet cells [39]. Interestingly, some UPR components can also play protein folding-unrelated roles in the pathogenesis of IBD. Grp94, an ER-stress induced ER chaperone, was overexpressed on the apical surface of IECs in the ileum of patients with Crohn's ileitis, and facilitated the invasion of pathogenic adherent-invasive *Escherichia coli* as a host cell receptor [40].

In addition to studies on specific UPR components, increased evidence suggests that ER stress may be an important contributing factor to IBD pathogenesis. Patients with active Crohn's disease and ulcerative colitis exhibit signs of ER stress in their ileal and/or colonic epithelium with active disease [28, 41-43]. Interestingly, the unaffected mucosal tissue of patients with ulcerative colitis showed reduced eIF2α phosphorylation, suggesting an impaired integrated stress response that can be activated by various cellular stress conditions including ER stress, viral infection, inflammation, oxidative stress, amino acid deficiency, and heme depletion) [9, 44]. Mice expressing a mutant *Muc2* gene displayed increased accumulation of MUC2 precursor in the ER of goblet cells, reduced mucin secretion, an impaired mucus layer, and activation of innate as well as adaptive immunity

with an induced Th17 response in the colon, which is similar to that of ulcerative colitis in human patients [42, 45]. Importantly, some ulcerative colitis patients also exhibited accumulation of MUC2 precursors in the ER of colonic goblet cells, suggesting that protein folding defect is physiologically relevant to goblet cell pathology in ulcerative colitis [37]. Later, the same group showed that IL10, an anti-inflammatory cytokine crucial for intestinal homeostasis, alleviates ER stress and promotes mucin secretion in goblet cells [46].

IECs including Paneth and goblet cells play a crucial role in maintaining intestinal homeostasis as an interface between the microbiota and the immune system. The failure of IECs to control inflammatory responses may dramatically contribute to IBD pathogenesis. Previous studies indicate that cells with a high load of protein folding and secretion are sensitive to altered ER homeostasis and this can induce inflammatory gene expression [47]. Environmental signals including bacterial molecules, bile salts, and cholinergic stimuli as well as host innate and adaptive immunity stimulate the synthesis, maturation, and secretion of multiple antimicrobial peptides and mucins through the activation of signal transduction pathways including the NF-κB signaling [48-50]. The induced production of antimicrobial peptides and mucins can overwhelm protein secretory capacity of Paneth and goblet cells, respectively. On the other hand, exposure to inflammatory stimuli cause ER stress, although the precise mechanism(s) is not well understood. Upon exposure to high levels of exogenous antigens, inflammatory cytokines as well as luminal toxins and reactive oxidative/nitrosative species in the intestinal lumen, IECs may require efficient UPR signaling, including ER chaperone induction, to survive the heavy burden of protein folding and secretion. In contrast, chronic ER stress and a

defective UPR may impair the function and homeostasis of IECs and lead to intestinal inflammation. The observation that ER stress markers are induced in the intestinal epithelia of patients with Crohn's disease and ulcerative colitis, as well as human genetic evidence and animal studies suggest that perturbed ER homeostasis due to environmental stress or genetic lesions in IECs may contribute to the pathogenesis of IBD.

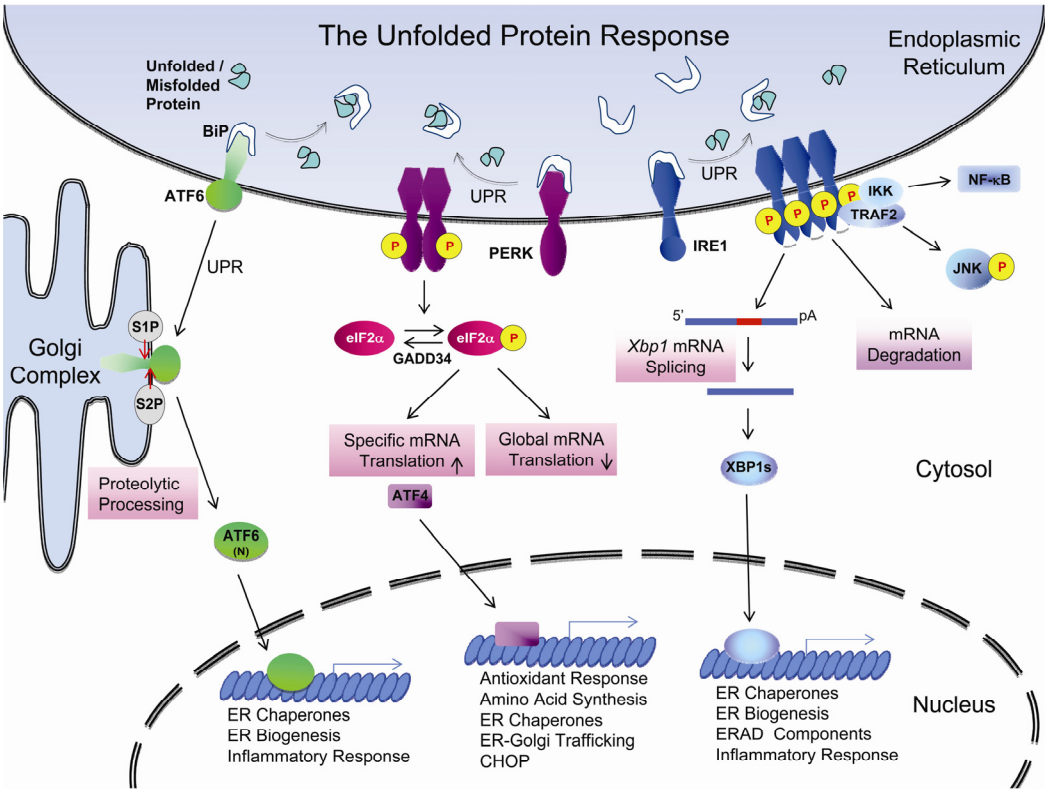

Figure 1-1: Unfolded protein response in mammals. See main text for details.

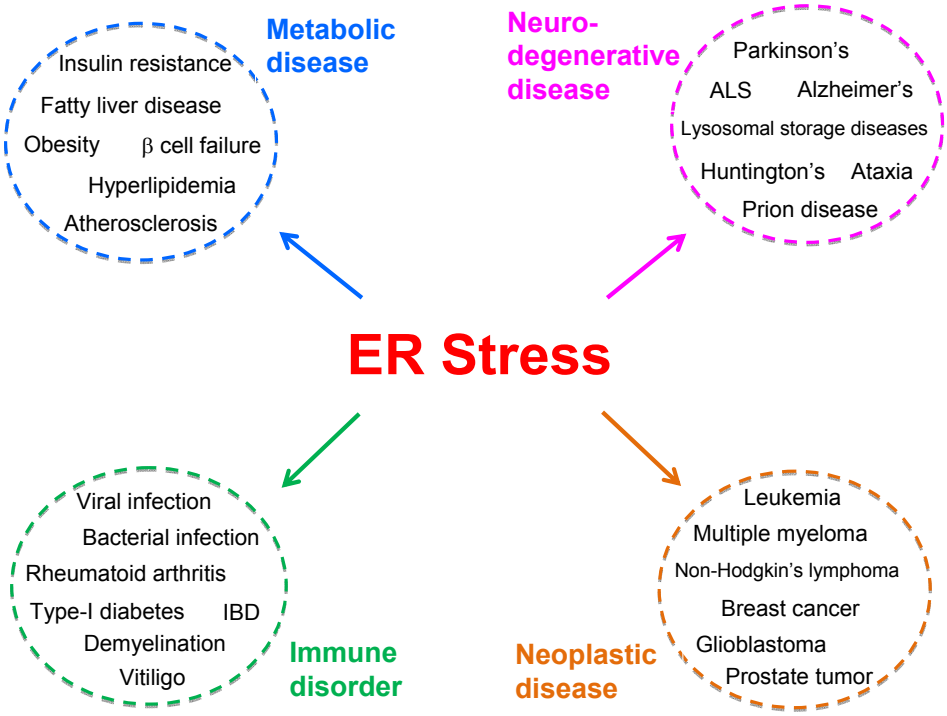

Figure 1-2: Endoplasmic reticulum stress in human disease. A number of human diseases are associated with ER protein folding defects and the unfolded protein response. The studies of ER stress and the UPR should enhance our understanding of the pathogenesis and open up new avenues to therapeutics.

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CHAPTER II

PKR PROTECTS COLONIC EPITHELIUM AGAINST COLITIS THROUGH THE UNFOLDED PROTEIN RRESPONSE AND PRO-SURVIVAL SIGNALING

Abstract

BACKGROUND & AIMS: The dsRNA-activated protein kinase (PKR) phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF2α), a global regulator of protein synthesis in mammals. In addition, PKR activates several signal transduction pathways including STAT3 and AKT. PKR is activated by a number of inflammatory stimuli that are induced in the inflamed intestine. In this study, we intend to determine the role of PKR in colonic epithelial cells during experimental colitis in mice. METHODS: Age- and sex- matched $PKR^{+/+,+/-}$ and $PKR^{-/-}$ littermate mice were reconstituted with wild-type bone marrow cells and subjected to dextran sodium sulfate (DSS)-induced colitis. RESULTS: $PKR^{-/-}$ mice displayed more severe clinical and histological manifestations upon DSS colitis compared with their $PKR^{+/+,+/-}$ littermates. In response to DSS colitis, the colonic epithelial cells of $PKR^{-/-}$ mice exhibited impaired activation of the UPR signaling, including eIF2α phosphorylation, ER chaperone response, and ER-associated degradation (ERAD) components, as well as anti-oxidative stress response. In addition, the phosphorylation of STAT3 and AKT, that are protective against epithelial

cell death and colonic inflammation, was also impaired in the colonic epithelial cells of *PKR*$^{-/-}$ mice upon DSS colitis. CONCLUSIONS: These data demonstrate that PKR is a physiologically relevant transducer of inflammatory response signaling in colonic epithelial cells. PKR may promote the homeostasis and survival of IECs through eIF2α-mediated UPR activation, as well as the activation of STAT3 and AKT pathways. In the absence of PKR, the survival and proliferation of IECs was impaired, thus exacerbating intestinal inflammation.

Keywords: PKR, DSS colitis, UPR, pro-survival signaling

Introduction

The endoplasmic reticulum (ER) in eukaryotic cells is the site for the folding, assembly, modification and maturation of polypeptides destined for secretion, membranes, and intracellular organelles. Protein folding in the ER is exquisitely sensitive to multiple environmental alterations and cellular disturbances including ER Ca^{2+} depletion, oxidative stress and inflammatory stimuli. Disrupted ER protein folding homeostasis leads to accumulation of unfolded/misfolded proteins in the ER lumen, a condition termed ER stress. To cope with this cellular stress and restore ER homeostasis, eukaryotic cells have evolved the unfolded protein response (UPR). In mammalian cells, three ER-localized stress sensors signal the UPR: inositol-requiring kinase 1 (IRE1), activating transcription factor 6 (ATF6) and PKR-like ER kinase (PERK). The UPR protein sensors are activated in response to ER stress and signal downstream pathways that control transcriptional, translational and posttranslational processes. During ER

stress, the ER chaperone BiP releases from the luminal domain of PERK, that is activated through dimerization and *trans*-autophophorylation. Activated PERK then phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF2α) to reduce global protein synthesis. At the same time, phosphorylated eIF2α induces the translation of mRNA encoding activating transcription factor 4 (ATF4), that subsequently transactivates genes encoding other UPR-associated transcription factors, ER protein-folding machinery, and components of ER-associated protein degradation (ERAD), and promotes cellular homeostasis by inducing the biosynthesis and transport of amino acids, and spurring the anti-oxidative stress response.[1]

In addition to PERK, three cytosolic eIF2α kinases exist in mammals, including the double-stranded RNA-activated protein kinase (PKR)[2]. PKR was originally identified and characterized as a pathogen sensor and mediator of the interferon response to protect the host from viral infection. PKR-mediated eIF2α phosphorylation is a strategy to shut down viral protein synthesis in host cells.[3] However, in contexts other than viral infection, induction of downstream UPR signaling and inhibition of global translation through the same eIF2α phosphorylation by PKR activation may be crucial for ER homeostasis in response to various environmental alterations.[2] Indeed a number of reports have demonstrated that PKR does signal protection to ER stress. In addition to dsRNA, PKR can be activated by other inflammatory signals including LPS and TNF-α, as well as oxidative stress. When activated, PKR induces or acts in conjugation with a number of signal transduction pathways, including p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinases (JNK), signal transducer and activator of transcription 3 (STAT3), and Akt/Protein kinase B.[4] Therefore, PKR functions as a regulatory hub that

coordinates inflammatory response signaling, pathogen sensing and UPR signaling in mammalian cells.

Inflammatory bowel diseases (IBD) represent a set of inflammatory conditions in the gastrointestinal tract. Although IBDs were discovered dozens of years ago, the etiology of IBD is still unclear. Recently, some studies linked ER stress and UPR signaling to the pathogenesis of IBD, by showing that patients with active Crohn's disease and ulcerative colitis exhibit signs of ER stress in their ileal and/or colonic epithelium.[5-8] Genes that regulate ER homeostasis and the UPR, including *XBP1*, *AGR2*, and *ORMDL3*, have been identified to associate with Crohn's disease and/or ulcerative colitis.[11-13] The murine and human intestinal epithelium contains Paneth and goblet cells, that have massive secretory functions and play essential roles in intestinal homeostasis and host defense. Intestinal epithelial cell (IEC)-specific deletion of *XBP1* caused Paneth cell death and spontaneous inflammation in murine ileum.[8] AGR2 is a protein disulfide isomerase that is required for the folding, modification and secretion of mucin2 in the murine intestine.[14] The deletion of *AGR2* caused abnormalities in both Paneth cells and goblet cells and increased ER stress in IECs.[15] A previous study suggested that PKR may regulate the differentiation of a rat intestinal epithelial cell line by promoting the activity of alkaline phosphatase.[9] However, the role of PKR in intestinal inflammation has not been characterized. Here we show that the deletion of PKR increases sensitivity to dextran sodium sulfate (DSS)-induced colitis in mice, due to reduced induction of adaptive UPR signaling and pro-survival signaling including the activation of STAT3 in colonic epithelial cells during inflammation.

Material and Methods

Mice

All mice were housed with 12-hour light and 12-hour dark cycles in the Unit for Laboratory Animal Medicine (ULAM) at the University of Michigan Medical Center with free access to water and standard rodent chow. All animal care and procedures were conducted following the protocols and guidelines approved by the University of Michigan Committee on the Use and Care of Animals (UCUCA).

Generation of Bone Marrow Chimeras

8-week old $PKR^{-/-}$ and $PKR^{+/+,+/-}$ littermate mice[10] were lethally irradiated with 950 rad ionizing irradiation. After two hours, 5×10^6 bone marrow cells isolated from the tibias and femurs of wild-type mice were injected into the lethally irradiated recipient mice through the tail vein. After transplantation, mice were treated with 6-week of antibiotics in their drinking water and allowed to recover for another 2 weeks prior to treatment with DSS to induced colitis.

DSS-induced Colitis

For DSS-induced acute colitis, $PKR^{-/-}$ and $PKR^{+/+,+/-}$ mice reconstituted with wild-type bone marrow cells received 3% (w/v) DSS (MW 36,000-50,000; MP Biomedicals) in drinking water for 7 days. Body weight change and rectal bleeding were monitored daily. After DSS administration, the mice were euthanized, colon length was measured,

colon sections and colonic epithelial cells were isolated for histological and biochemical analyses.

Isolation of Colonic Epithelial Cells

The entire colons were cut open longitudinally, then feces was removed by washing with ice-cold PBS. Colons were then cut into 2-3 mm pieces and incubated in Ca^{2+}, Mg^{2+}- free PBS buffer containing 10 mM EDTA in 50 mL conical tube at 4°C for 1hr with gentle rotation. Then the tubes were rigorously shaken to elute the epithelium from colon sections. The supernatant was removed and sieved through a cell strainer (500 um; Fisherbrand). The flow-through was centrifuged; the resulting cell pellet was washed twice in ice-cold PBS and snap-frozen in liquid nitrogen for protein and RNA extraction. The purity of isolated colonic epithelial cells was confirmed by FACS and Q-RT-PCR. Trypan blue staining confirmed the presence of > 85% viable epithelial cells after the 2-hr isolation procedure.

Quantitative Real-Time PCR

RNA from isolated colonic epithelial cells and IEC-6 cells were extracted by using RNeasy kit (Qiagen); RNA from 5mm distal colons was isolated by using TRIzol reagent (Invitrogen). Q-RT-PCR was performed as previously described.[11] Q-RT-PCR results were normalized to the levels of 18S rRNA or the mRNA encoding glyceraldehyde 3-phosphate dehydrogenase. Primer sequences are listed in Supplemental Table 1.

Western Blotting

The isolated colonic epithelial cells were lysed in RIPA buffer for protein extraction. Protein content was measured by Protein Assay (Bio-rad), and protein samples were analyzed by 10–20% reducing SDS/PAGE (Tris-HCl precast gels, Bio-rad) and were detected with the following antibodies: anti-GRP94/BiP (Stressgen), anti-phosphor-PERK (Cell Signaling Technology), anti-ATF4 (Santa Cruz Biotechnology), anti-CHOP (Santa Cruz Biotechnology), anti-XBP1 (Santa Cruz Biotechnology), anti-LC3 (Novus Biologicals), and anti-glyceraldehyde 3-phosphate dehydrogenase (Millipore), anti-phosphor-STAT3 (Cell Signaling Technology). After over-night incubation of primary antibodies at 4°C, the membranes were washed and then incubated with secondary HRP-coupled antibodies (GE Healthcare) and developed with chemiluminescent detection system (GE Healthcare).

Immunohistochemical Staining

Colonic tissues were fixed overnight in 10% formalin, embedded in paraffin, and cut into 5 μm sections. Paraffin sections were rehydrated, heat-induced antigen retrieval was then performed in 10 mM sodium citrate, 0.05% Tween-20, pH 6.0 for 10 minutes. - Primary antibodies: anti-BiP antibody (Santa Cruz Biotechnology) at a dilution of 1:200; anti-ATF4 antibody (LifeSpan BioSciences) at a dilution of 1:200; anti-phosphor-STAT3 antibody (LifeSpan BioSciences) at a dilution of 1:200. Hematoxylin was performed for counterstaining.

Histological Scoring

Colonic tissues were fixed overnight in 10% formalin, embedded in paraffin, and cut into 5μm sections for hematoxylin and eosin (H&E) staining using a standard protocol. H&E stained sections were scored in a blinded manner by a licensed animal pathologist for the amount of tissue damage and inflammatory cell infiltration as previously described.[12] The damaged area involved was scored on a scale of 0 to 4: 0 = no involvement, 1 ≤ 25%, 2 ≤ 50%, 3 ≤ 75%, 4 ≤ 100%. Inflammatory cell infiltration were scored on a scale of 0 to 3: 0 = occasional inflammatory cells in the lamina propria, 1 = increased numbers of inflammatory cells in the lamina propria, 2 = confluence inflammatory cells, extending into the submucosa, and 3 = transmural extension of the infiltrate.

Statistical Analysis

All data are presented as means ± SEM. Statistical significance of the difference between groups was analyzed using a Student t-test. $P < 0.05$ was considered significant.

Results

Mice with PKR deletion in non-hematopoietic cells are more sensitive to DSS-induced colitis.

In the absence of inflammatory insults, the colon of *PKR*$^{-/-}$ mice is morphologically indistinguishable to that of their wild-type littermates. To analyze a possible requirement for PKR in IECs to prevent IBD, lethally irradiated age- and sex-matched *PKR*$^{+/+,+/-}$ and *PKR*$^{-/-}$ littermate mice were reconstituted with wild-type bone marrow (Figure 2-1A) and then fed 3% DSS in the drinking water for 7 days to induce

colitis. *PKR*$^{-/-}$ mice showed more severe clinical manifestations, as demonstrated by body weight loss, rectal bleeding and colon shortening upon DSS challenge (Figure 2-1B-D). In addition, *PKR*$^{-/-}$ mice displayed significantly higher levels of damaged area in the epithelium and inflammatory cell infiltration in both the proximal and distal colon (Figure 2-2A and B). Consistent with the histological observations, the expression of pro-inflammatory genes *Il1β*, *Tnfα* and *Inos* were dramatically induced in the colon of *PKR*$^{-/-}$ mice upon DSS colitis compared with their wild-type and heterozygous littermates (Figure 2-3). In contrast, deletion of *PKR* in the hematopoietic compartment does not affect the response to DSS colitis in mice (Figure 2-1E).

UPR induction is impaired in colonic epithelial cells of PKR$^{-/-}$ mice.

Our previous studies demonstrated that UPR molecules, e.g. BiP and ATF4, are induced in colonic epithelium in response to DSS colitis (data shown in later chapters). Given the role of PKR as an inducer of UPR signaling, we then examined whether UPR activation is impaired in *PKR*$^{-/-}$ colonic epithelial cells during inflammation. Western blotting demonstrated that colonic epithelial cells of *PKR*$^{-/-}$ mice exhibited reduced levels of eIF2α phosphorylation and its downstream target ATF4 compared with their wild-type littermate mice. The induction of the ER chaperones BiP and GRP94 was also impaired at both the protein and RNA levels (Figure 2-4A, B and C). In addition to ER chaperones, the induction of a number of UPR pathways were compromised in *PKR*$^{-/-}$ mice upon DSS colitis, including the UPR transcription factors XBP1s and ATF6, ER protein foldng machinary ERp72, Ero1α and PDI, ERAD components Herpud1 and Wfs1, and the antioxidative stress response nuclear factor-erythroid 2-related factor 2 (Nrf2).

Previous studies demonstrated that these adaptive UPR signaling pathways are essential during intestinal inflammation. The UPR-induced chaperone response in colonic epithelium has been shown to protect against DSS colitis in mice.[13] Loss of Nrf2 increases susceptibility to DSS colitis, probably a consequence of enhanced oxidative damage and pro-inflammatory responses in the colonic mucosa.[14] Therefore, PKR may protect colonic epithelial cells during inflammation through the induction of UPR signaling. In the absence of PKR, UPR signaling was impaired and may exacerbate ER stress and induce apoptosis. Consistently, the cleavage of caspase-3 was highly activated in colonic epitheial cells of *PKR*$^{-/-}$ mice after DSS challenge (Figure 2-4A).

Pro-survival signaling is compromised in absence of PKR during DSS colitis.

Given that PKR is an upstream inducer of STAT3 and Akt phosphorylation,[4] we next examined whether the activation of these two signaling molecules was impaired in colonic epithelium of *PKR*$^{-/-}$ mice. Western blotting demonstrated that colonic epithelial cells isolated from *PKR*$^{-/-}$ mice challenged with DSS exhibited reduced phosphorylation of STAT3 and Akt compared with their wild-type littermate mice (Figure 2-4A). Consistently, the nuclear staining of phospho-STAT3 in the epithelial cells was diminished in the colon of *PKR*$^{-/-}$ mice (Figure 2-4C).

Discussion

In this study we demonstrate that PKR is a physiologically relevant activator of UPR signaling in colonic epithelium upon inflammation. In response to DSS colitis, PKR promoted homeostasis and survival of IECs through eIF2α-mediated UPR signaling.

PKR$^{-/-}$ IECs showed reduced phosphorylation of eIF2α upon colitis, consistent with the diminished eIF2α phosphorylation observed in *PKR*$^{-/-}$ mouse embryo fibroblasts (MEFs) in response to inflammatory stimuli including IFNα/β.[15] Reduced eIF2α phosphorylation in *PKR*$^{-/-}$ IECs upon colitis impaired the activation of UPR, e.g. transcription factors, chaperone response, ERAD components and anti-oxidative stress response. This is consistent with our observations that mice expressing a non-phosphorylatable eIF2α in IECs are susceptible to DSS colitis and display diminished UPR induction in their colonic epithelial cells upon colitis (data shown in later chapters). The UPR-activated transcription factors XBP1, ATF6α and ATF4 are essential transactivators of UPR gene induction in response to ER stress. Mice deficient in XBP1 in the intestinal epithelium were senstive to DSS colitis, although the underlying mechansim is not yet clear.[8] ATF6α is activated upon ER stress by sequential cleavage by proteases S1P and S2P, yielding an active ATF6α p50 that functions as a potent activator of a number of ER chaperones including BiP, GRP94 and P58^{IPK}. The hypomorphic mutation of S1P-encoding gene *Mbtps1* impaired ATF6α-dependent induction of chaperone response, and increased susceptibility to experimental colitis in mice.[13] ATF4 is induced at both the transcriptional and translational levels during ER stress and functions as an essential global UPR transactivator.[2] In addition, ATF4 is activated by oxidative stress and plays an important role in the anti-oxidative stress response.[16] Although there is little information about the role of ATF4 in IBD, it is possible that ATF4 is protective against ER stress and oxidative stress in IECs during intestinal inflammation. The importance of UPR-induced ER chaperones in DSS colitis is directly supported by our study using a murine model deficient in P58^{IPK}, a heat shock 40 kDa protein that normally resides in

association with the ER chaperone BiP in the ER lumen and promotes proper protein folding.[17] *P58*$^{IPK-/-}$ mice are highly susceptible to DSS colitis, probably due to unresolved ER stress and selective activation of pro-apoptotic UPR signaling in colonic IECs during inflammation (data shown in later chapters). There are few studies examining the role of ERAD pathways in IBD. However, given the function of ERAD in eliminating unfolded/misfolded protein and alleviating ER stress, it is possible that ERAD also impacts IEC function during intestinal inflammation. Therefore, the compromised induction of adaptive UPR pathways may exacerbate the dysfunction of colonic IECs in *PKR*$^{-/-}$ mice upon DSS challenge, and consequently worsen the symptoms of colitis.

PKR has been shown to physically interact with STAT3 and induce the phosphorylation at Tyr705 and Ser727, which is required for DNA binding and the transcriptional activation function of STAT3 upon PDGF stimulation.[18] STAT3 is a pleiotropic transcription factor with crucial roles in a variety of cellular processes including proliferation and differentiation. Previous studies demonstrated that STAT3 activity is increased by phosphorylation in IECs during colonic inflammation, and transactivates the genes responsible for the stress response, apoptosis and wound healing in IECs. IEC-specific deletion of *STAT3* increased the susceptibility to DSS colitis.[19] Similarly, the pleiotropic protein kinase AKT functions to inhibit IEC apoptosis and promote wound healing during intestinal inflammation.[20] Therefore, compromised activation of STAT3 and AKT in *PKR*$^{-/-}$ mice may impair function and survival of IECs, thus promoting intestinal inflammation upon DSS challenge.

Several previous studies have linked PKR to apoptotic cell death. In NIH3T3 and COS-1 cells, TNFα induces apoptosis through PKR-mediated eIF2α phosphorylation.[21]

PKR$^{-/-}$ MEFs were resistant to apoptosis upon challenge with dsRNA, TNFα, and lipopolysaccharide.[22] Another study showed that dsRNA-activated PKR induced the expression of pro-apoptotic molecules including Fas and Bax in 3T3 L1 cells.[23] In macrophages, cholesterol/7-ketocholesterol-induced oxidative stress and activated the PKR-CHOP pathway that was required for the downstream apoptotic response.[24] However, all of these studies were performed *in vitro* and the cells analyzed were very different from colonic epithelial cells at both cell biological and physiological levels. Therefore, it seems the protective role of PKR in colonic epithelial cells may be both cell-type and/or disease-specific.

Another study published at the same time by Rath et al. using mice with deletion of the C-terminal catalytic kinase domain of PKR suggest that PKR is detrimental to the host in the context of injury that results in colonic inflammation.[25, 26] These findings conflict with our results that show *Pkr*$^{-/-}$ mice with deletion of the N-terminal dsRNA binding domain display impaired survival of intestinal epithelial cells and greater intestinal inflammation in an experimental model of colitis.[27, 28] It is possible that the potential differences between the N-terminal and C-terminal murine deletion mutants of PKR are responsible for this discrepancy. However, the level of expression of the N-terminal truncated PKR kinase domain is extremely low.[29] In addition, our studies detected reduced eIF2α phosphorylation in the *Pkr*-null mice and this was not increased upon DSS treatment. The findings indicate that induction of the endogenous truncated allele does not contribute to eIF2α phosphorylation in this model. Importantly, another factor that contributes to DSS sensitivity is the genetic background.[30, 31] Although both Cao et al. and Rath et al. used mixed backgrounds for the *Pkr*-deleted mice (Cao et al.

used a 129Sv(ev)/C57BL/6 background and Rath et al. used a 129/terSv/BALB/C background), Cao et al. used littermate controls to rule out effects of genetic background and maternal host microflora. In contrast, Rath et al. used inbred BALB/C mice as controls. Numerous reports demonstrate that genetic background as well as maternal microflora significantly impact progression of intestinal inflammation in animal models.[32-36] Therefore, it is important to consider the impact of genetic background and maternal microflora contributions in these inflammatory responses. In addition, Cao et al. used bone marrow chimeras for the DSS colitis experiments to study effects in intestinal epithelial cells, while Rath et al. used whole body C-terminal deleted *Pkr*. Although many cellular pathways are intact in cells expressing C-terminal deleted PKR, some signal transduction pathways including phosphoinositide 3-kinase (PI3K) signaling are impaired in absence of the C-terminal catalytic domain.[25, 29, 35] Given the important role of the PI3K family in immunity, it is possible that the protective effect of the C-terminal deletion in PKR during DSS colitis is partially due to a reduced inflammatory response from the immune cells.[36, 37]

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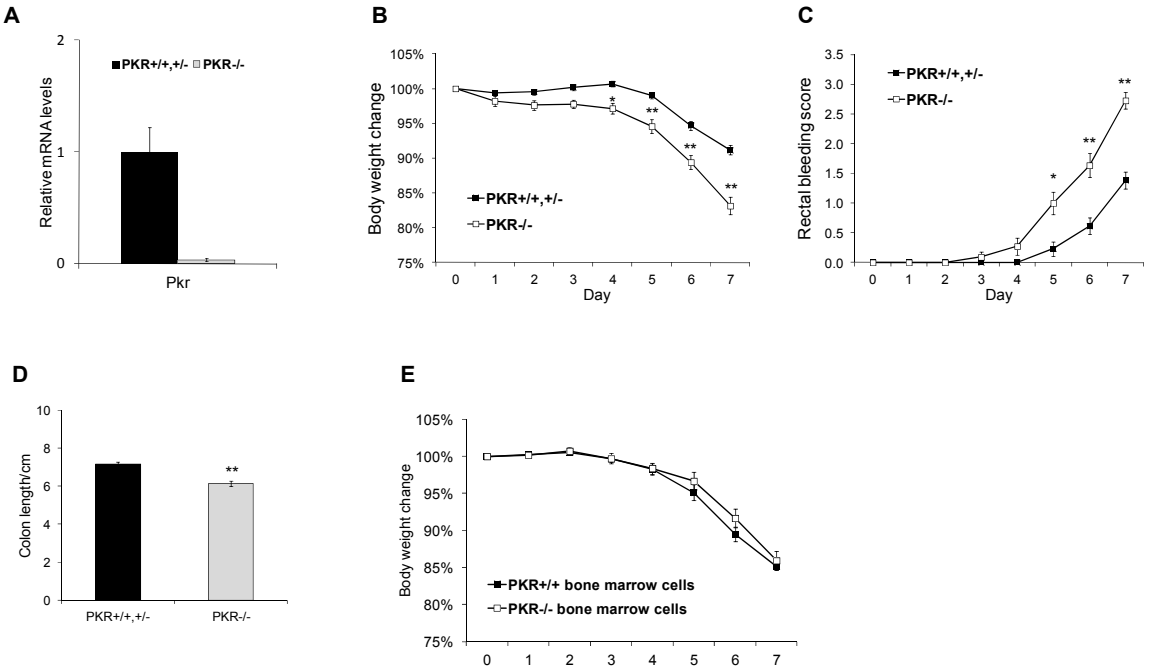

Figure 2-1: *PKR*$^{-/-}$ mice are more sensitive to DSS-induced colitis. *PKR*$^{-/-}$ and *PKR* $^{+/+,+/-}$ littermates were reconstituted with wild-type bone marrow cells. After 8 weeks, the mice were fed 3% DSS in drinking water for 7 days, their body weight and rectal bleeding were monitored daily. (*A*) 8-week after bone marrow transplant, colonic IECs were isolated from *PKR*$^{-/-}$ and *PKR* $^{+/+,+/-}$ mice for measure of *Pkr* mRNA. (*B*) *PKR*$^{-/-}$ mice show more significant body weight loss during DSS colitis. (*C*) *PKR*$^{-/-}$ mice show more severe rectal bleeding during DSS colitis. (*D*) *PKR*$^{-/-}$ mice show more severe colon shortening after 7-day of DSS administration. *PKR* $^{+/+,+/-}$: n = 13; *PKR*$^{-/-}$: n = 11; *p<0.05, **p<0.01. (*E*) Wild-type mice were reconstituted with *PKR*$^{-/-}$ or *PKR* $^{+/+}$ bone marrow cells. After 8 weeks, the mice were fed 3% DSS in the drinking water for 7 days, their body weight was monitored daily.

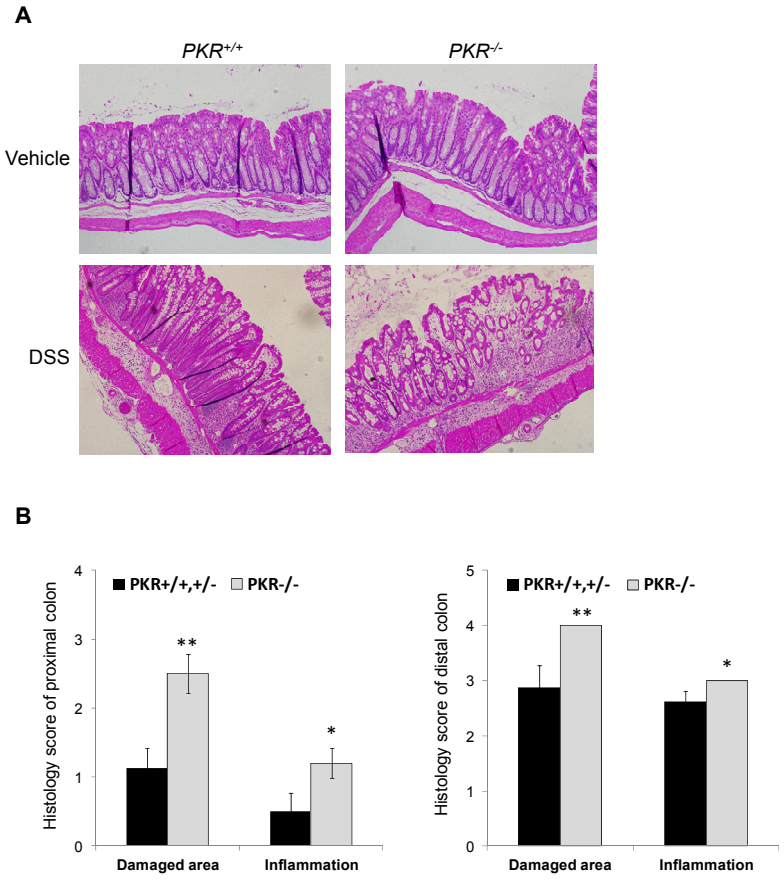

Figure 2-2: *PKR*$^{-/-}$ mice show more severe histological manifestations after 7-day of DSS administration. *PKR*$^{-/-}$ and *PKR*$^{+/+,+/-}$ littermates reconstituted with wild-type bone marrow cells were fed 3% DSS in drinking water for 7 days. *(A)* After DSS administration, the colons were isolated and fixed for H&E staining. Representative images were shown (100x). *(B)* Histological scores including damage area involved and inflammatory cell infiltration taken from *PKR*$^{-/-}$ and *PKR*$^{+/+,+/-}$ littermates with DSS colitis. *PKR*$^{+/+,+/-}$: n = 8; *PKR*$^{-/-}$: n = 10; *p<0.05, **p<0.01.

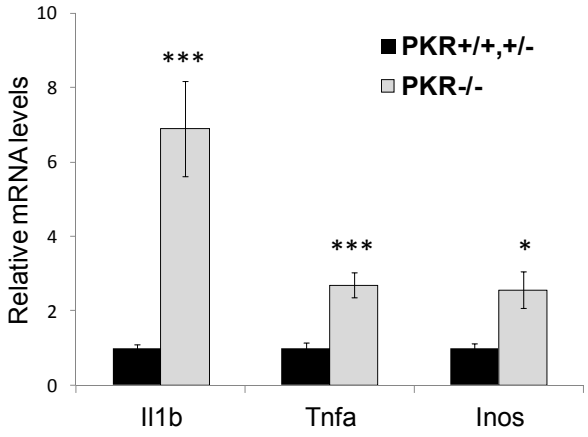

Figure 2-3: *PKR*$^{-/-}$ mice show hyperactivated inflammatory response in the colon upon DSS administration. *PKR*$^{-/-}$ and *PKR* $^{+/+,+/-}$ littermates with wild-type bone marrow cells were fed 3% DSS in drinking water for 7 days. After DSS administration, the mice were euthanized and colonic epithelial cells were collected for RNA extraction, cDNA synthesis and Q-RT-PCR. The mRNA levels were normalized to the expression of *Gapdh*. *PKR*$^{+/+,+/-}$: n = 6; *PKR*$^{-/-}$: n = 5; *p<0.05, **p<0.01, ***p<0.001.

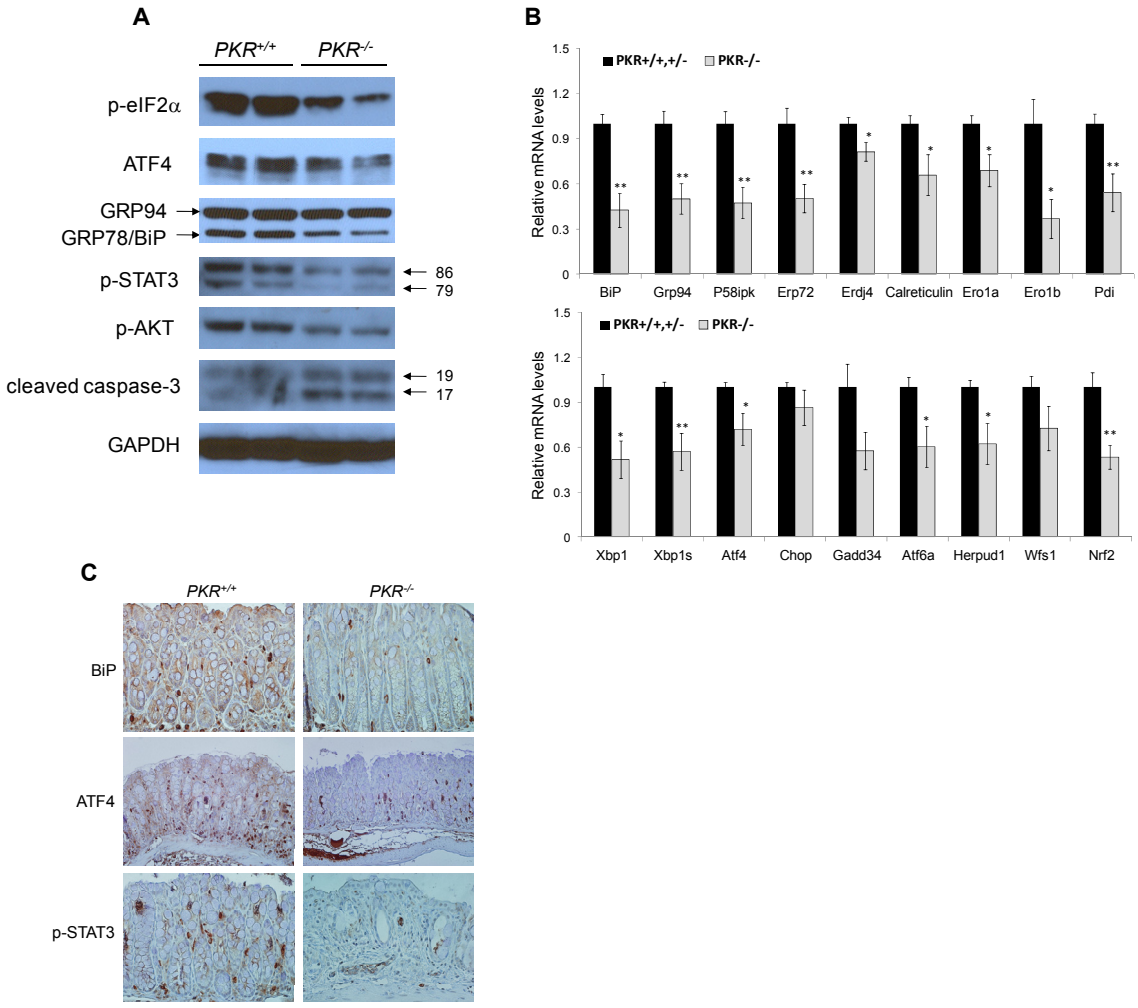

Figure 2-4: Activation of UPR signaling and pro-survival pathways is impaired in *PKR*$^{-/-}$ colonic epithelial cells upon DSS colitis. *PKR*$^{-/-}$ and *PKR* $^{+/+,+/-}$ littermates with wild-type bone marrow cells were fed 3% DSS in drinking water for 6 days. *(A)* After DSS administration, the mice were euthanized and the colonic epithelial cells were isolated for protein extraction and immunoblotting. *(B)* After DSS administration, the mice were euthanized and the colonic epithelial cells were isolated for RNA extraction, cDNA synthesis and Q-RT-PCR. The mRNA levels were normalized to the expression of *Gapdh*. *(C)* After DSS administration, the mice were euthanized and the colons were isolated, fixed and paraffin embedded for immunohistochemical staining of BiP, ATF4 and phospho-STAT3 (200x or 400x). *PKR*$^{+/+,+/-}$: n = 6; *PKR*$^{-/-}$: n = 6; *p<0.05, **p<0.01.

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CHAPTER III

THE UNFOLDED PROTEIN RESPONSE AND CHEMICAL CHAPERONES REDUCE PROTEIN MISFOLDING AND COLITIS IN MICE

Abstract

BACKGROUND & AIMS: Endoplasmic reticulum (ER) stress has been associated with development of inflammatory bowel disease (IBD). We examined the effects of ER stress-induced chaperone response and the orally active, chemical chaperones tauroursodeoxycholate (TUDCA) and 4-phenylbutyrate (PBA), which facilitate protein folding and reduce ER stress, in mice with colitis. METHODS: We used dextran sulfate sodium (DSS) to induce colitis in mice that do not express the transcription factor ATF6α or the protein chaperone $P58^{IPK}$. We examined the effects of TUDCA and PBA in cultured intestinal epithelial cells (IECs), in wild-type, $P58^{IPK-/-}$ and $Atf6\alpha^{-/-}$ mice with colitis, and in $Il10^{-/-}$ mice. RESULTS: $P58^{IPK-/-}$ and $Atf6\alpha^{-/-}$ mice developed more severe colitis following administration of DSS than wild-type mice. IECs from $P58^{IPK-/-}$ mice had excessive ER stress, and apoptotic signaling was activated in IECs from $Atf6\alpha^{-/-}$ mice. Inflammatory stimuli induced ER stress signals in cultured IECs, which were reduced by incubation with TUDCA or PBA. Oral administration of either PBA or TUDCA reduced features of DSS-induced acute and chronic colitis in wild-type mice, the colitis that develops in $Il10^{-/-}$ mice, and DSS-induced colitis in $P58^{IPK-/-}$ and $Atf6\alpha^{-/-}$ mice. Reduced signs of colonic inflammation in these mice were associated with significantly decreased ER stress in colonic

epithelial cells. CONCLUSIONS: The UPR induces expression of genes that encode chaperones involved in ER protein folding; these factors prevent induction of colitis in mice. Chemical chaperones such as TUDCA and PBA alleviate different forms of colitis in mice and might be developed for treatment of inflammatory bowel diseases.

Keywords: IBD, mouse model, ulcerative colitis, therapeutic agent

Introduction

The processes of protein folding, modification and maturation in the ER are sensitive to environmental changes and multiple cellular disturbances including ER Ca^{2+} depletion, defective glycosylation, metabolic stimuli, altered redox status, energy deprivation, inflammatory stimuli, as well as increased protein secretion. When ER protein folding is perturbed, or when cells are stimulated to secrete large amounts of protein, unfolded/misfolded proteins accumulate in the ER lumen, a condition called ER stress [1]. In order to restore ER function and improve protein-folding homeostasis (proteostasis), eukaryotes evolved the unfolded protein response (UPR). In mammalian cells, the UPR is initiated by three ER-localized transmembrane protein sensors: activating transcription factor 6α (ATF6α), inositol-requiring kinase 1α (IRE1α), and PKR-like ER kinase (PERK) [2-4]. UPR signaling can lead to either adaptation or apoptosis. In the adaptive UPR, ER protein folding is remodeled through transactivation of genes encoding ER chaperones, ER trafficking machinery and ER associated protein degradation (ERAD) and by eIF2α phosphorylation-mediated global translation attenuation [5-8]. Alternatively, prolonged and/or severe ER stress leads to the activation of the pro-apoptotic UPR, including the transcription factor CHOP and the IRE1α-activated cJun N-terminal kinase (JNK) pathway [9]. Moreover, chronic ER stress impairs cellular homeostasis through energy depletion, leakage of ER Ca^{2+},

mitochondrial damage, oxidative stress, and activation of caspases [10]. Therefore, persistent protein misfolding in response to chronic environmental stress and/or ineffective adaptive UPR signaling can compromise cell function and homeostasis and induce apoptosis [11].

Recent studies link ER stress to the pathogenesis of IBD. For example, patients with active Crohn's disease and ulcerative colitis exhibit signs of ER stress in their ileal and/or colonic epithelium [12-15]. In addition, human genetic studies of IBD have identified primary genetic abnormalities in several genes including *XBP1*, *AGR2*, and *ORMDL3* that encode proteins associated with ER stress [15-19]. Previous studies indicate that cells with a high load of protein folding and secretion are sensitive to altered ER homeostasis and this can induce inflammatory response gene expression [5, 20, 21]. Intestinal microbiota and their molecules stimulate IECs to increase secretion of mucins and antimicrobial peptides that can overwhelm their protein secretory capacity. On the other hand, exposure to inflammatory stimuli can cause ER stress, although the precise mechanism is not well understood [21]. Upon exposure to high levels of exogenous antigens and inflammatory cytokines in the intestinal lumen, IECs may require efficient UPR-mediated ER chaperone induction to survive the heavy burden of protein folding and secretion.

In this study, we show that protein misfolding in the ER caused by deletion of the ER co-chaperone gene *P58^{IPK}/Dnajc3* exacerbates experimental colitis in mice. ATF6α is a potent transcriptional activator for a number of ER chaperone genes including *Bip*, *Grp94* and *P58^{IPK}* in many cell types [2-4]. Although whole body deletion of *Atf6α* does not generate an obvious phenotype under normal conditions, it is required for cells to survive chemical-induced ER stress [8]. We found that in the absence of P58^{IPK} or ATF6α mice are sensitive to colitis and exhibit reduced induction of ER chaperone genes and hyperactivation of pro-apoptotic UPR signaling in colonic IECs. The chemical chaperones TUDCA and PBA are FDA-approved bioactive small

molecules that function to facilitate protein folding and reduce ER stress both *in vitro* and *in vivo* by stabilizing protein-folding intermediates and preventing protein aggregation [22-28]. In this study, we show that oral delivery of either TUDCA or PBA dramatically decreases the clinical, histological and biochemical signs of inflammation in both innate immunity- and T cell-dependent colitis through reducing ER stress signaling in colonic IECs.

Material and Methods

Mice

Atf6α$^{-/-}$ and *P58*$^{IPK-/-}$ mice (C57BL/6J background) were described previously [8, 29]. Wild-type C57BL/6J mice were purchased from The Jackson Laboratory. All animal care and procedures were conducted according to the protocols and guidelines approved by the University of Michigan's UCUCA and SBMRI's IACUC.

Cell Culture

IEC-6 cells (passage 8-10) were kindly provided by Dr. Linda Samuelson in the Department of Physiology, at the University of Michigan Medical Center. The cells were maintained in Dulbecco's modified Eagle's medium containing 4.5 mg/mL glucose and supplemented with 2 mmol/L L-glutamine, 10 mmol/L HEPES, 100 mg/ml streptomycin, and 100 U/ml penicillin. Cells were seeded 5×10^5 cells/well in 12-well plates and all experiments were performed at 1 day after cultures reached confluence. Cells were treated with 100 ng/mL rat TNFα (R&D Systems, Minneapolis, MN), 100 ng/mL rat MCP-1 (R&D Systems), 25 ng/mL rat IL-1β (R&D Systems) and 5 mM PBA (Scandinavian Formulas, Inc.) or TUDCA (EMD Chemicals USA).

Generation of Bone Marrow Chimeras

Littermate mice of *Atf6α*$^{+/+}$, *Atf6α*$^{+/-}$, *Atf6α*$^{-/-}$ and *P58*$^{IPK+/+}$, *P58*$^{IPK+/-}$, and *P58*$^{IPK-/-}$ were lethally irradiated with 950 rad ionizing irradiation. After two hours, 5×10^6 bone marrow cells isolated from the tibias and femurs of wild-type mice were injected into the lethally irradiated recipient mice through the tail vein. After transplantation, mice were treated with 4 weeks of antibiotics in their drinking water and allowed to recover for another 4 weeks prior to treatment with DSS to induce colitis.

DSS-induced Colitis, Piroxicam-induced Colitis in Il10$^{-/-}$ Mice, and Drug Administration

For acute colitis, mice received 2-3% (w/v) DSS (MW 36,000-50,000; MP Biomedicals, Solon, OH) in drinking water for indicated days. To develop long-lasting chronic DSS colitis, 8-week old C57BL/6J mice received 3 cycles of 2% (w/v) DSS (7 days DSS, 14 days water) as previously described [30].

Six-month old *Il10*$^{-/-}$ mice (C57BL/6J background) were fed 200 ppm piroxicam, a non-steroidal anti-inflammatory drug (NSAID), in the diet for two weeks to induce colitis as previously described [31]. The mice were subject to PBA/TUDCA treatment for three weeks after feeding of prixicam-diet.

For administration of TUDCA (EMD Chemicals USA, Gibbstown, NJ) and PBA (Scandinavian Formulas, Inc., Sellersville, PA), the sodium salt was dissolved in sterile PBS (Invitrogen, Carlsbad, CA) in a volume of 200 mL and given to mice by gavage, or dissolved in drinking water at a concentration of 2 mg/mL. Water intake measurements showed that *Il10*$^{-/-}$ mice with colitis consumed an average of 2.6 mL of water with or without drug every day. Therefore, each *Il10*$^{-/-}$ mouse received an average of 5.2 mg of PBA or TUDCA per day.

Isolation of Colonic Epithelial Cells

Colons were cut open longitudinally and feces were removed by washing with ice-cold PBS. Colons were then cut into 2-3 mm pieces and incubated in Ca^{2+}, Mg^{2+}- free PBS buffer containing 10 mM EDTA in a 50 mL conical tube at 4°C for 1hr with gentle rotation. Then the tubes were rigorously shaken to elute the epithelium from colon sections. The supernatant was removed and sieved through a cell strainer (500 um; Fisher HealthCare, Houston, TX). The flow-through was centrifuged; the resulting cell pellet was washed twice in ice-cold PBS (32). The purity of isolated colonocytes was determined by flow cytometry using an antibody against murine epithelial cell adhesion molecule from BioLegend (San Diego, CA, USA) following the manufacturer's protocol. Trypan blue staining confirmed the presence of > 85% viable epithelial cells after the 2-hr isolation procedure. Isolated cells were snap-frozen in liquid nitrogen for protein and RNA extraction.

Quantitative Real-Time PCR

RNAs from isolated murine colonic epithelial cells and IEC-6 cells were extracted by using RNeasy kit (Qiagen, Valencia, CA); RNA from 5 mm distal colons was isolated by using TRIzol reagent (Invitrogen, Carlsbad, CA). Q-RT-PCR was performed as previously described [33, 49]. Q-RT-PCR results were normalized to the level of 18S rRNA or mRNA encoding glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primer sequences are listed in Table 3-1.

Western Blotting

The isolated colonic epithelial cells were lysed in RIPA buffer for protein extraction. Protein content was measured by Protein Assay (Bio-Rad, Hercules, CA), and protein samples were analyzed by 10–20% reducing SDS/PAGE (Tris-HCl precast gels, Bio-Rad) and were

detected with the following antibodies: anti-GRP94/BiP (Enzo Life Sciences International, INC., Plymouth Meeting, PA), anti-phosphor-PERK (Cell Signaling Technology, Danvers, MA), anti-ATF4 (Santa Cruz Biotechnology, Santa Cruz CA), anti-CHOP (Santa Cruz Biotechnology), anti-XBP1 (Santa Cruz Biotechnology), anti-Bim (Cell Signaling Technology), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Millipore Corporation, Billerica, MA), and anti-tubulin (Cell Signaling Technology). After over-night incubation of primary antibodies at 4°C, the membranes were washed and then incubated with secondary HRP-coupled antibodies (GE Healthcare, Piscataway, NJ) and developed with chemiluminescent detection system (GE Healthcare).

Immunohistochemical Staining

Colonic tissues were fixed overnight in 10% formalin, embedded in paraffin, and cut into 5 μm sections. Paraffin-embedded tissue sections were rehydrated, heat-induced antigen retrieval was performed either in 10 mM sodium citrate, 0.05% Tween-20, pH 6.0, or in 10 mM Tris, 1 mM EDTA, pH 8.0, for 10 minutes. Primary antibodies: anti-BiP antibody (Santa Cruz Biotechnology) at a dilution of 1:200 at room temperature; anti-CHOP antibody (Santa Cruz Biotechnology) at a dilution of 1:50 at room temperature; anti-XBP1 antibody (BioLegend) at a dilution of 1:50 overnight at 4°C. Hematoxylin was performed for counterstaining.

Histological Scoring

Hematoxylin and eosin (H&E) stained colon sections were scored in a blinded manner by board certified veterinary pathologists as previously described by Berg et al [31], Otuska et al [34], and Lee et al [56].

Results

DSS colitis induces ER stress in colonic epithelium.

To characterize the role of ER stress and the UPR in IECs during intestinal inflammation, we utilized the dextran sodium sulfate (DSS)-induced colitis murine model. C57BL/6J mice at 8 weeks of age were fed 3% DSS in their drinking water for 3 or 5 days to develop colitis in the large intestine. The purity of isolated colonic epithelial cells was higher than 95%, measured by flow cytometry using an antibody against murine epithelial cell adhesion molecule (EpCAM, Figure 3-8A). Analysis of UPR markers, including the ER chaperone BiP, phosphorylation of the ER stress sensor PERK, and the UPR transcription factors ATF4, CHOP and spliced XBP1 (XBP1s), demonstrated a time-dependent induction in colonic epithelium that coincided with progression of DSS colitis. A similar mRNA induction pattern of UPR genes was observed by Q-RT-PCR analysis and immunohistochemical (IHC) staining (Figure 3-8C, D). The ER stress induction in the DSS-induced colitis model is similar to that observed in intestinal tissues from patients with active IBD [12-15], suggesting that this experimental colitis model is valid for studies of ER stress and the UPR in IECs upon intestinal inflammation.

The ER co-chaperone P58^{IPK} protects from DSS colitis.

To determine the requirement for proper protein folding in the ER for IEC function, we analyzed mice with deletion in *P58^{IPK}*. P58^{IPK} is a heat shock 40 kDa protein that belongs to the DNAJ chaperone family, and resides in the ER lumen in association with the ER chaperone BiP and promotes proper protein folding [36-38]. The UPR induces transcription of *P58^{IPK}*, while cells and mice deleted in *P58^{IPK}* display slight protein misfolding and are sensitive to ER stress [29, 36]. The role of P58^{IPK} in colonic epithelia was studied using bone marrow chimeras of *P58$^{IPK+/-}$* and *P58$^{IPK-/-}$* mice to exclude the impact of *P58^{IPK}* deletion in hematopoietic cells. Upon challenge

with DSS, $P58^{IPK-/-}$ mice displayed severe body weight loss, rectal bleeding and shortening of the large intestine (Figure 3-1A, B; Figure 3-9A). Consistently, the $P58^{IPK-/-}$ mice showed significantly more severe mucosal damage, loss of goblet cells, and inflammatory cell infiltration in the colon compared with their heterozygous littermates (Figure 3-1C, E; Figure 3-9B). After DSS challenge, immunoblots demonstrated upregulation of ER stress markers BiP and phosphor-IRE1α in isolated colonic IECs from $P58^{IPK-/-}$ mice compared to both $P58^{IPK+/+}$ and $P58^{IPK+/-}$ mice. The pro-apoptotic transcription factor CHOP was also induced in $P58^{IPK-/-}$ colonic IECs before and after DSS treatment (Figure 3-1E). Consistently, IHC indicated increased expression of CHOP in $P58^{IPK-/-}$ colonic epithelium with DSS colitis (Figure 3-1F). Bim, a BH3-only member of the Bcl-2 family that is transactivated by CHOP, plays a critical role in ER stress-induced apoptosis [39]. Upon DSS challenge, Bim and cleaved caspase-3 were highly induced in the colonic epithelium of $P58^{IPK-/-}$ mice compared to both $P58^{IPK+/+}$ and $P58^{IPK+/-}$ mice (Figure 3-1E). These data suggest the elevated susceptibility of $P58^{IPK-/-}$ mice to DSS colitis is due to a hyperactivated ER stress response and pro-apoptotic UPR signaling, including CHOP and Bim, in colonic IECs during inflammation.

ATF6α-mediated ER chaperone induction protects against DSS colitis.

To further elucidate the role of ER chaperones induced during intestinal inflammation, we studied mice with a deletion in *Atf6α*, the master regulator of ER chaperone gene expression. The colon of $Atf6\alpha^{-/-}$ mice is indistinguishable from wild-type, as indicated by H&E and periodic acid-Schiff (PAS) staining (Figure 3-10A). Age and gender-matched $Atf6\alpha^{+/+}$, $Atf6\alpha^{+/-}$ and $Atf6\alpha^{-/-}$ littermate mice were reconstituted with wild-type bone marrow cells. The genetic ablation of *Atf6α* exacerbated symptoms of DSS colitis, including severe body weight loss, rectal bleeding (Figure 3-2A, B), and significantly greater mucosal damage, goblet cell loss, and macrophage

infiltration in the colon (Figure 3-2C, D; Figure 3-10B). *Atf6α*$^{-/-}$ mice displayed reduced expression of ER chaperone genes including *Bip, Grp94* and *P58*^{IPK} in both protein and mRNA levels (Figure 3-2E; Figure 3-10C), indicating the adaptive UPR signaling in colonic IECs of *Atf6α*$^{-/-}$ mice is compromised in response to intestinal inflammation. In contrast, the pro-apoptotic IRE1α-JNK pathway was induced in inflamed colonic epithelium of *Atf6α*$^{-/-}$ mice (Figure 3-2E). After colitis induction, the apoptotic markers cleaved caspase-3 and DNA fragmentation (by TUNEL staining) were also increased in colonic epithelium of *Atf6α*$^{-/-}$ mice compared to *Atf6α*$^{+/+}$ and *Atf6α*$^{+/-}$ mice (Figure 3-2E; Figure 3-10D, E). Consistently, IHC showed reduced expression of the ER chaperone BiP and increased expression of the pro-apoptotic transcription factor CHOP in *Atf6α*$^{-/-}$ colonic epithelium (Figure 3-2F). These data are consistent with the notion that ATF6α is an important transactivator of ER chaperone genes in colonic IECs during colitis. The impaired ER chaperone induction in *Atf6α*$^{-/-}$ mice leads to unresolved ER stress, which induces pro-apoptotic UPR signaling in colonic IECs and exacerbates DSS colitis.

TUDCA and PBA alleviate inflammation-induced ER stress in an intestinal epithelial cell line.

Given the protective role of the ER chaperone response in IECs against intestinal inflammation, we tested whether the chemical chaperones TUDCA and PBA can reduce ER stress in IECs and alleviate colitis in mice. We first analyzed the effect of PBA and TUDCA in the non-transformed rat IEC line IEC-6 that was treated with physiologically relevant stimuli to cause ER stress. The inflammatory cytokines TNFα, MCP-1 and IL-1β are highly upregulated in animal models of enterocolitis and IBD patients. We found that a combined cocktail of TNFα, MCP-1, and IL-1β induce ER stress in IEC-6 cells, as monitored by the upregulation of ER stress markers BiP and CHOP (Figure 3-3A). Prior treatment and co-treatment of IEC-6 cells with

either PBA or TUDCA reduced CHOP and BiP expression in response to the inflammatory stimuli (Figure 3-3A). These data indicate that pro-inflammatory cytokines induce ER stress in IECs *in vitro*, and this cellular stress can be mitigated by either PBA or TUDCA treatment.

TUDCA and PBA alleviate signs of DSS colitis by reducing ER stress signaling in colonic epithelial cells.

The therapeutic potential of chemical chaperones was tested in a prevention paradigm in the DSS colitis murine model. C57BL/6J mice were fed 2.5% DSS in their drinking water for 8 days. TUDCA was administrated orally by gavage at 500mg/kg body weight per day (single dose) throughout the whole period. Compared to mice subjected to DSS challenge only, feeding of TUDCA significantly ameliorated the symptoms of DSS colitis, as indicated by the lower clinical scores (Figure 3-3B, C; Figure 3-11). TUDCA dramatically reduced the histological manifestations of DSS colitis (Figure 3-3D, E). In parallel, the expression of pro-inflammatory cytokines IL-1β and TNFα in the colon was significantly reduced by TUDCA feeding (Figure 3-3F). Additionally, the induction of oxidative/nitrosative stress and apoptotic signaling was inhibited by TUDCA treatment, as indicated by diminished expression of genes encoding iNOS, NOX2 and, Bim (Figure 3-3F). As expected, the induction of ER stress markers BiP, $P58^{IPK}$, CHOP, GADD34 and ERO1α in colonic IECs was considerably reduced in mice fed with TUDCA during the induction of DSS colitis (Figure 3-3F), which is consistent with the observations in IEC-6 cells treated with inflammatory signals and TUDCA. Similarly, feeding of PBA dramatically ameliorated the symptoms of DSS colitis (Figure 3-4A-E). ER stress induction in colonic epithelium was significantly reduced upon PBA administration during the induction of DSS colitis (Figure 3-4F).

TUDCA and PBA reverse DSS-induced chronic colitis.

Then we examined whether the chemical chaperones can reverse the symptoms of chronic colitis. The mice were fed with 3 cycles of 2% DSS in drinking water, then received 300 mg/kg body weight TUDCA daily (double dose, 150 mg/kg body weight per dose) by oral administration for 10 days. Dramatically, the histology scores of the large intestine, including damaged area involved, ulceration, mucodepletion of glands, and inflammatory cell infiltration, were significantly reduced after the administration of TUDCA compared with the controls (Figure 3-5A, B). For PBA delivery, C57BL/6J mice with chronic colitis were fed with 500 mg/kg body weight PBA daily (double dose, 250 mg/kg body weight per dose) for 10 days. PBA-treated mice also showed a similar recovery from chronic colitis (Figure 3-5C, D).

TUDCA and PBA complement the requirement for P58^{IPK} and ATF6α in preventing DSS colitis.

To further explore the molecular mechanisms of how chemical chaperones function in alleviating intestinal inflammation, we tested the two compounds on DSS colitis in the *P58^{IPK}* or *Atf6α* mice. Where *P58$^{IPK-/-}$* and *Atf6α$^{-/-}$* mice were more susceptible to DSS colitis, as shown in Figures 1 and 2, feeding of TUDCA or PBA reduced the clinical and histological scores of *P58$^{IPK-/-}$* and *Atf6α$^{-/-}$* mice to similar levels as those of their littermate controls (Figure 3-6A-F). These data indicate that the chemical chaperones can complement the requirement for molecular chaperones, correct the protein folding defects in animals with an impaired ER chaperone response and protect mice against intestinal inflammation.

TUDCA and PBA dramatically mitigate colitis in Il10$^{-/-}$ mice.

Since TUDCA and PBA can alleviate both acute and chronic colitis induced by DSS, we then examined whether the two chemical chaperones can ameliorate NSAID-induced colitis in *Il10*$^{-/-}$ mice, a T cell-dependent IBD model. *Il10*$^{-/-}$ mice with established colitis received either TUDCA or PBA in the drinking water at a concentration of 2mg/mL (5.2 mg/mouse per day) for three weeks. Dramatically, the histology scores of the large intestine were dramatically reduced after the administration of either TUDCA or PBA (Figure 3-7A upper and middle panel, 3-7B, C). Furthermore, trichome staining indicated that fibrosis in the large intestine was significantly reduced by the feeding of TUDCA or PBA (Figure 3-7A lower panel, 3-7D). In the colonic epithelia of *Il10*$^{-/-}$ mice with colitis, the induction of ER stress markers including BiP, phospho-eIF2α, and CHOP, mitochondrial UPR marker HSP60, as well as apoptotic markers including cleaved caspase-3/12 were considerably reduced after the treatment with TUDCA or PBA (Figure 3-7E). These data demonstrate that the chemical chaperones have potent anti-inflammatory and anti-fibrotic effects in the *Il10*$^{-/-}$ colitis model through the suppression of ER stress and cell death in colonic epithelium.

Discussions

Recent studies indicate that inflammatory conditions in the gastrointestinal tract can activate the UPR in IECs [12-15]. However, it is unknown whether these pathway(s) function to disrupt cellular homeostasis and induce apoptosis, or to restore ER function and prevent cell death. Previous studies demonstrated that P58^{IPK} binds to newly synthesized secretory proteins in the ER, and promotes protein folding/maturation in cells [34, 36-38]. Consistent with a role for P58^{IPK} in reducing protein misfolding in the cell, we show that P58^{IPK} prevents IECs dysfunction and progression of DSS colitis. During the development of colitis, *P58*$^{IPK-/-}$ mice display increased expression of the pro-apoptotic factor CHOP and reduced expression of the pro-survival protein

Bcl2 in IECs, due to an unresolved/prolonged ER stress. CHOP is a major cell death-inducing factor during the UPR [9, 15] and has been shown to exacerbate colitis in mice [41]. Previous studies demonstrated that a hypomorphic mutation in the gene encoding S1P in mice enhances the sensitivity to DSS colitis [42]. However, given that S1P targets several ER stress-induced bZIP transcription factors including Luman, OASIS, and CREBH, as well as the SREBPs [43], it was not clear whether the increased susceptibility is attributed to reduced activation of ATF6 or other transcription factors. In this study, we show that colonic IECs from *Atf6α*$^{-/-}$ mice have reduced adaptive ER chaperone expression and increased pro-apoptotic UPR signaling including the IRE1α-JNK pathway [9, 15]. In mice with an IEC-specific deletion of *Xbp1*, hyper-phosphorylated IRE1α activates JNK and induces spontaneous inflammation in the ileum [15, 44]. Therefore, compromised ER chaperone expression, by loss of either an ER chaperone itself or the upstream transactivator, leads to unresolved ER stress and activation of pro-apoptotic signaling, and therefore impairs cell function and exacerbates inflammation. In these experiments, we used bone marrow chimeras to exclude the impact of *P58*^{IPK} or *Atf6α* deletion in hematopoietic cells including macrophages, neutrophils, T cells, and B cells during intestinal inflammation. However, bone marrow reconstitution is not able to replace lamina propria fibroblasts and smooth muscle cells in the gut, which may still contribute to colitis. Villin-*Cre*-directed conditional deletion models, if available, would be ideal for this study.

Consistent with the protective role of ER chaperones in IECs for intestinal homeostasis, we demonstrated that the chemical chaperones TUDCA and PBA, which promote ER homeostasis and increase ER folding capacity, reduce ER stress signaling in IECs and alleviate colitis in mice. Furthermore, we show that feeding of TUDCA or PBA corrected the defects in *P58*$^{IPK-/-}$ and *Atf6α*$^{-/-}$ mice with impaired ER chaperone induction during DSS colitis, suggesting

that TUDCA and PBA resolve intestinal inflammation due to their function in promoting protein folding and alleviating ER stress.

Recent studies showed that TUDCA inhibits the expression of UPR genes in an intestinal epithelial cell line induced by the widely used ER stressor tunicamycin [45]. In our study, we show that either TUDCA or PBA reduces ER stress gene expression in IECs induced by inflammatory stimuli that are physiologically relevant to IBD. More strikingly, the feeding of either TUDCA or PBA dramatically protected the intestinal mucosa in both a DSS-induced model and a T cell-dependent genetic model of colitis. This data suggest that TUDCA and PBA exert epithelial-protective effect during the intestinal inflammation that is predominated by either the innate or adaptive immune response in the mucosa.

The chemical chaperones TUDCA and PBA are FDA-approved drugs that have outstanding safety profiles in humans. TUDCA is safely used as a hepato-protective drug for the treatment of primary biliary cirrhosis [46]. PBA is approved for clinical use in urea-cycle disorders [47]. Both compounds are in clinical trials for the treatment of a number of diseases that are associated with protein misfolding in the ER, including cystic fibrosis, amyotrophic lateral sclerosis, spinal muscular atrophy, Huntington's disease and type 2 diabetes (http://clinicaltrialsfeeds.org/clinical-trials/results/term=TUDCA; http://clinicaltrialsfeeds.org/clinical-trials/results/intr=4-phenylbutyric+acid) [2, 48-51]. Current medications used to treat IBD, including steroids, immunosuppressants and biologics, have significant risks and side effects [52-54]. If efficacy can be demonstrated in IBD patients, given the safety profile and potential for oral delivery of TUDCA and PBA, this therapy could fill an important gap in our current therapeutic armamentarium. Ursodeoxycholate, the unconjugated bile salt of TUDCA, is a promising drug for chemoprevention of colorectal cancer [55]. While TUDCA can inhibit inflammation-induced ER stress in non-transformed IEC-6 cells, it

exacerbates ER stress in some colon cancer cell lines (unpublished results). It would be worthwhile to determine whether TUDCA is able to suppress the growth of carcinogenic colonocytes while protecting normal colonic epithelial cells against ER stress in patients with ulcerative colitis. Based on our findings in multiple murine models of colitis, the chemical chaperones TUDCA and PBA may warrant clinical investigation as a novel treatment for IBD.

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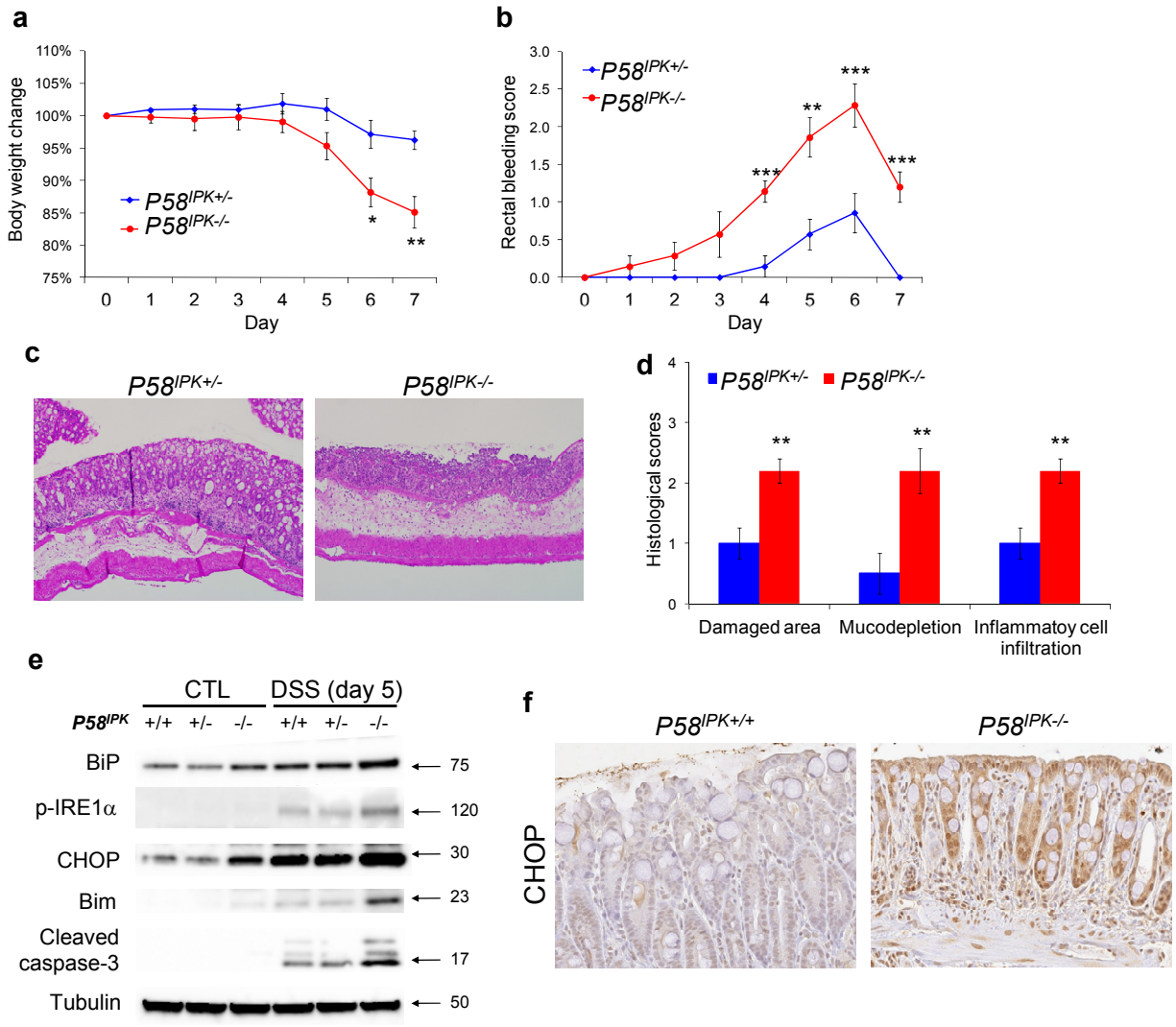

Figure 3-1: Loss of P58^{IPK} exacerbates DSS colitis in mice due to A hyperactivated pro-apoptotic UPR. *P58$^{IPK+/-}$* and *P58$^{IPK-/-}$* littermates with wild-type bone marrow cells were fed 2.5% DSS in drinking water for 5 days followed by 2 days of fresh water. Body weight (**a**) and rectal bleeding (**b**) were measured over 7 days. (**c**) After DSS administration, the colons were isolated and fixed for H&E staining. Representative images are shown (100x). (**d**) Histological scores were measured in mice with DSS colitis. (**e**) The pro-apoptotic transcription factor CHOP was induced while anti-apoptotic Bcl2 was reduced in *P58$^{IPK-/-}$* IECs. (**f**) IHC shows CHOP is induced in *P58$^{IPK-/-}$* colonic epithelium with DSS colitis. n = 7 for each group. *p<0.05, **p<0.01, ***p<0.001.

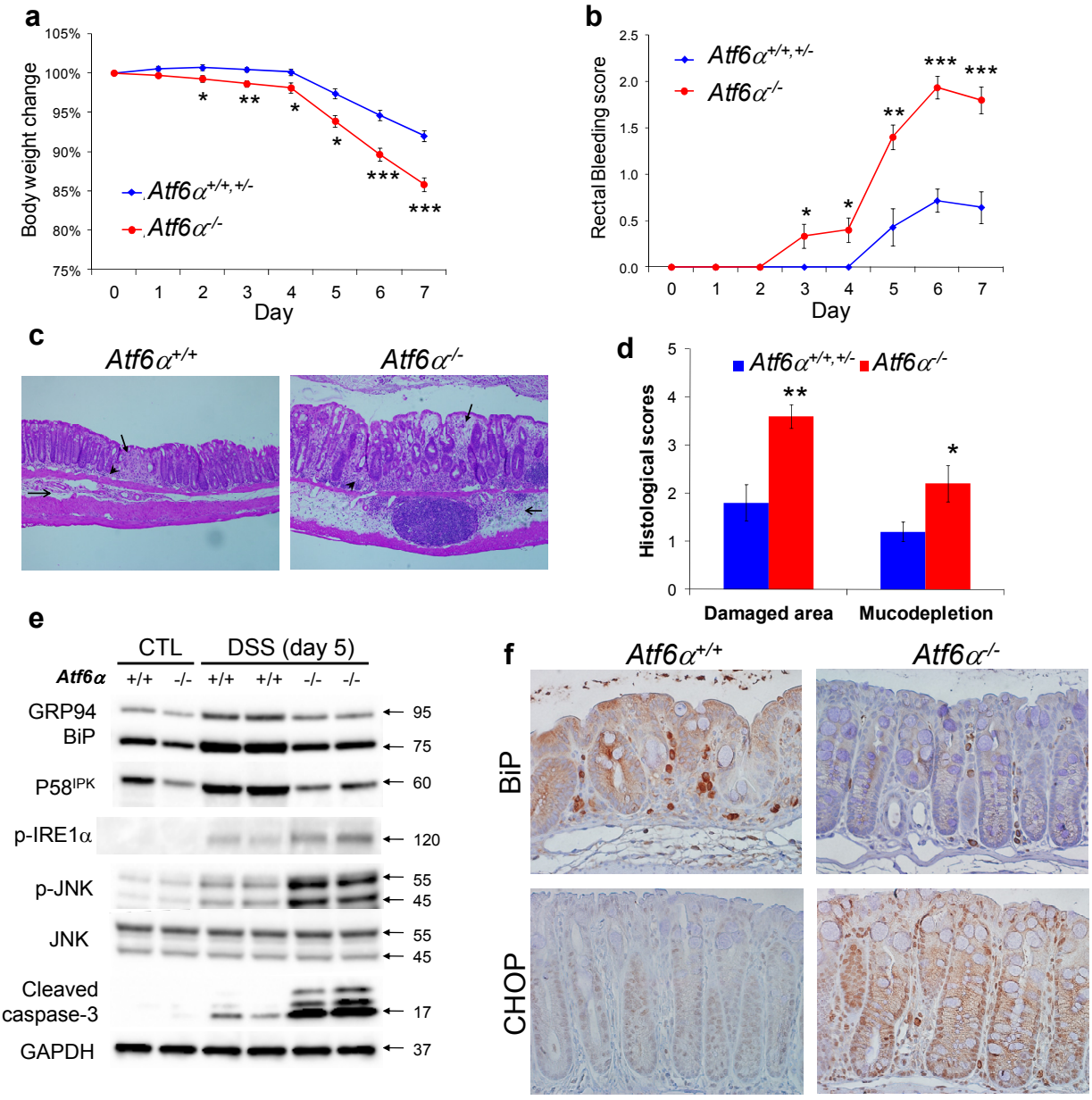

Figure 3-2: Loss of ATF6α exacerbates DSS colitis in mice due to defective ER chaperone induction and a hyperactivated pro-apoptotic UPR. *Atf6α*$^{+/+}$, *Atf6α*$^{+/-}$ and *Atf6α*$^{-/-}$ littermates with wild-type bone marrow cells were fed 3% DSS in drinking water for 5 days followed by 2 days of fresh water. Body weight (**a**) and rectal bleeding **(b)** were measured over 7 days. After DSS administration, the colons were isolated and fixed for H&E staining. Representative images are shown (**c**, 100x). (**d**) Histological scores were measured in mice with DSS colitis. (**e**) The expression of ER chaperones BiP, GRP94 and P58^{IPK} is reduced, while pro-apoptotic IRE1α-JNK pathway and caspase-3 are activated in *Atf6α*$^{-/-}$ IECs with DSS colitis. (**f**) IHC shows expression of the ER chaperone BiP is impaired while the pro-apoptotic transcription factor CHOP is increased in *Atf6α*$^{-/-}$ colonic epithelium with DSS colitis. n = 14 or 15. *p<0.05, **p<0.01, ***p<0.001.

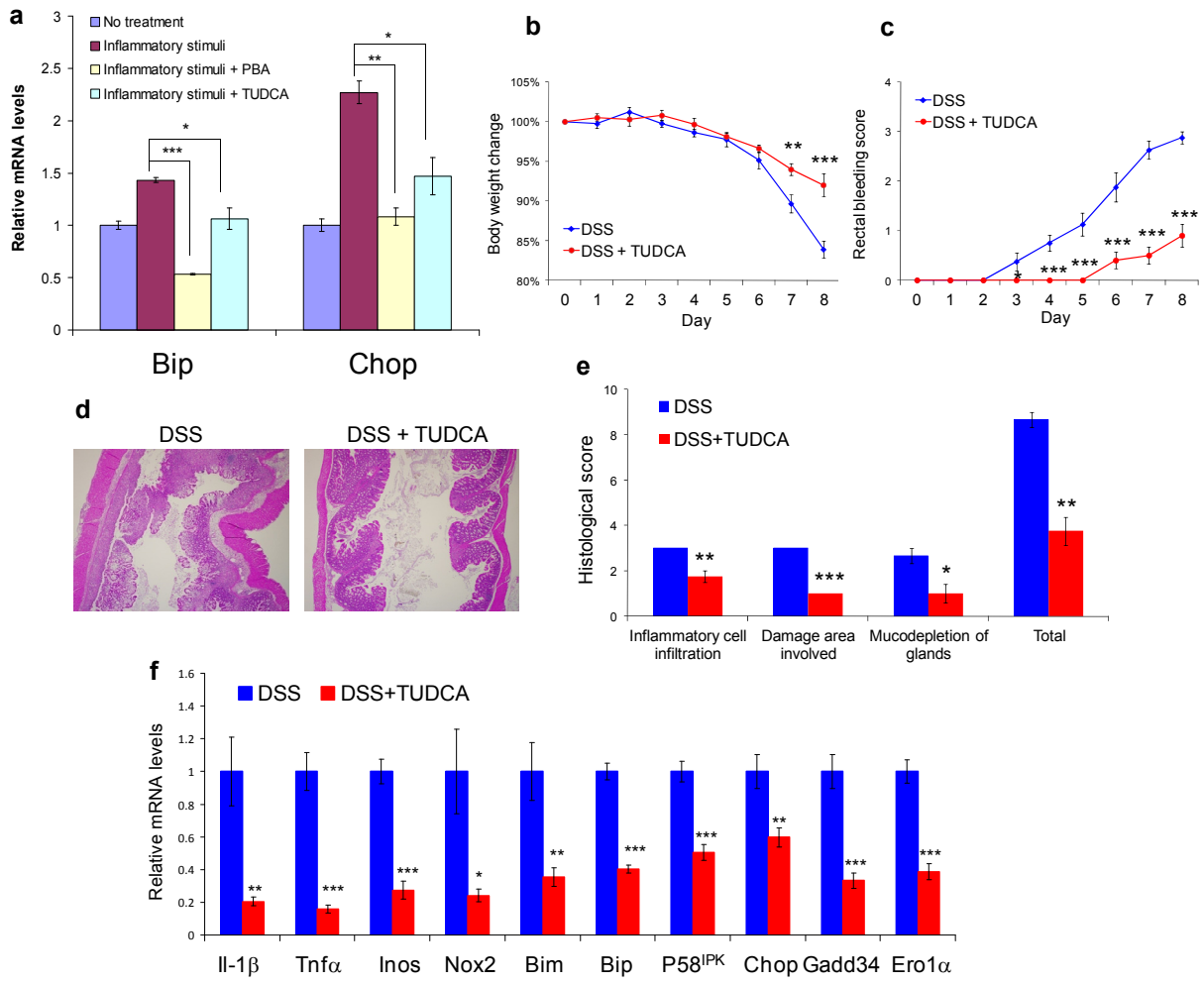

Figure 3-3: TUDCA and PBA alleviate inflammatory stimuli-induced ER stress in IECs *in vitro*; TUDCA ameliorates DSS colitis by reducing ER stress in colonic epithelium *in vivo*. (**a**) IEC-6 cells were treated with a combination of inflammatory cytokines (TNFα, MCP-1, and IL-1β) for 8h; or pre-treated with 5 mM TUDCA or PBA for 4h, followed by treatment with the same inflammatory signals with 5 mM TUDCA or PBA, for 8h. The cells were then collected for RNA extraction and Q-RT-PCR. The mRNA levels were normalized to the expression of 18S rRNA. Wild-type mice were fed 2.5% DSS in drinking water and received 500 mg/kg body weight TUDCA or the same amount of PBS without TUDCA daily by gavage (n = 8 or 10 per group). Body weight (**b**) and rectal bleeding (**c**) were measured over 8 days. (**d**) After DSS administration, the colons were isolated and fixed for H&E staining. Representative images are shown (40x). (**e**) Histological scores are shown from TUDCA-treated and control mice with DSS colitis. (**f**) Expression of genes associated with inflammation, oxidative stress, and apoptosis in colonic mucosa, as well as ER stress markers in colonic IECs is shown (normalized to the expression of *Gapdh*). n = 8 for each group; *p<0.05, **p<0.01, ***p<0.001.

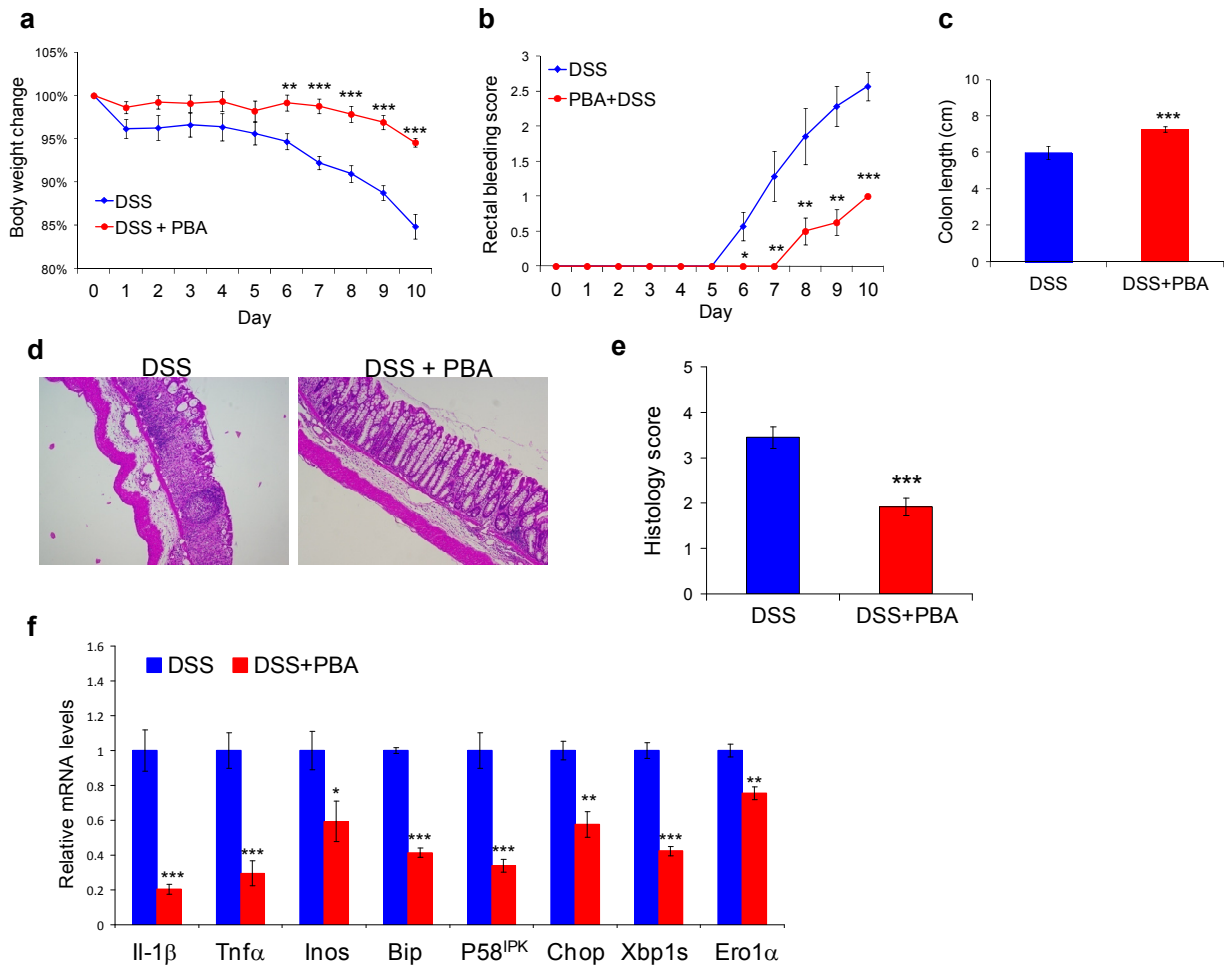

Figure 3-4: PBA alleviates DSS colitis by reducing ER stress in colonic epithelium. Wild-type mice were fed 2% DSS in drinking water and received 500 mg/kg body weight PBA or the same amount of PBS without PBA daily by gavage. Body weight **(a)** and rectal bleeding (**b**) were measured over 10 days. (**c**) Colon lengths were measured after DSS colitis. (**d**) After DSS administration, the colons were isolated and fixed for H&E staining. Representative images are shown (40x). (**e**) Histological scores are shown from control and PBA-treated mice with DSS colitis. (**f**) Expression of genes associated with inflammation, oxidative stress, and ER stress in colonic mucosa, as well as ER stress markers in colonic IECs is shown (normalized to the expression of *Gapdh*). n = 8 for each group; *p<0.05, **p<0.01, ***p<0.001.

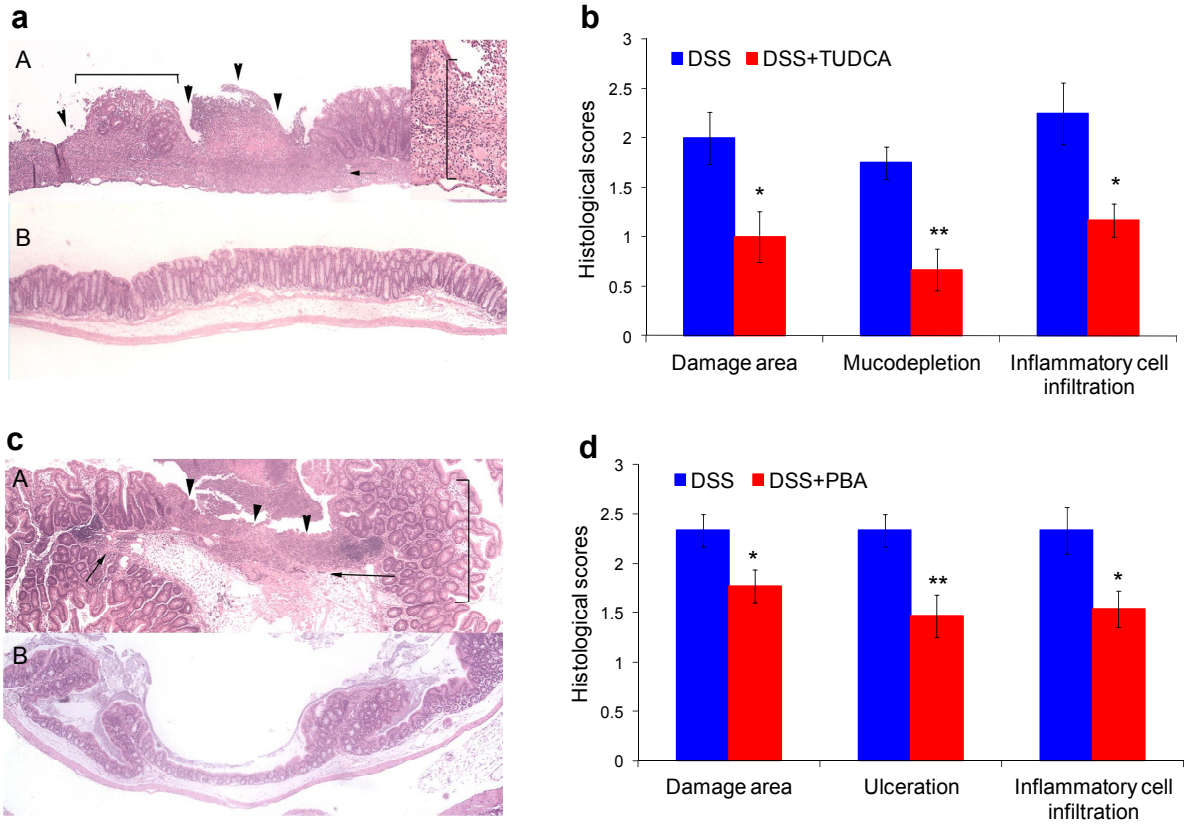

Figure 3-5: Either TUDCA or PBA mitigates inflammation in mice with chronic colitis. (**a**) Wild-type mice with established chronic DSS colitis received 300 mg/kg body weight TUDCA or the same amount of PBS without TUDCA (Control) daily by gavage for 10 days. A: Colon from a mouse with chronic colitis fed with PBS alone (Control) demonstrating severe epithelial ulceration (arrowheads), loss of goblet cell morphology (mucodepletion, bracket), and transmural inflammatory infiltrate (arrow). Inset demonstrates transmural inflammatory infiltrate at the edge of an ulcer (bracket). B: Mice with chronic colitis treated with TUDCA display reduced mucosal damage and inflammation. (40x; 100x for inset) (**b**) Histological scores including damaged area involved, mucodepletion of glands, and inflammatory cell infiltration are shown from TUDCA-treated and control mice with chronic DSS colitis. n = 6 or 8 for each group. (**c**) Wild-type mice with established chronic DSS colitis received 500 mg/kg body weight PBA or the same amount of PBS without PBA (Control) daily by gavage for 10 days. A: Cecum from a mouse with chronic colitis fed with PBS alone (Control) demonstrating severe ulceration (arrowheads), inflammatory infiltrates (arrows), and loss of goblet cell morphology (bracket). B. Cecum from a mouse with chronic colitis treated with PBA demonstrating reduced mucosal damage and inflammation. (40x) (**d**) Histological scores are shown from PBA-treated and control mice with chronic DSS colitis. n = 9 or 13 for each group. *$p<0.05$, **$p<0.01$.

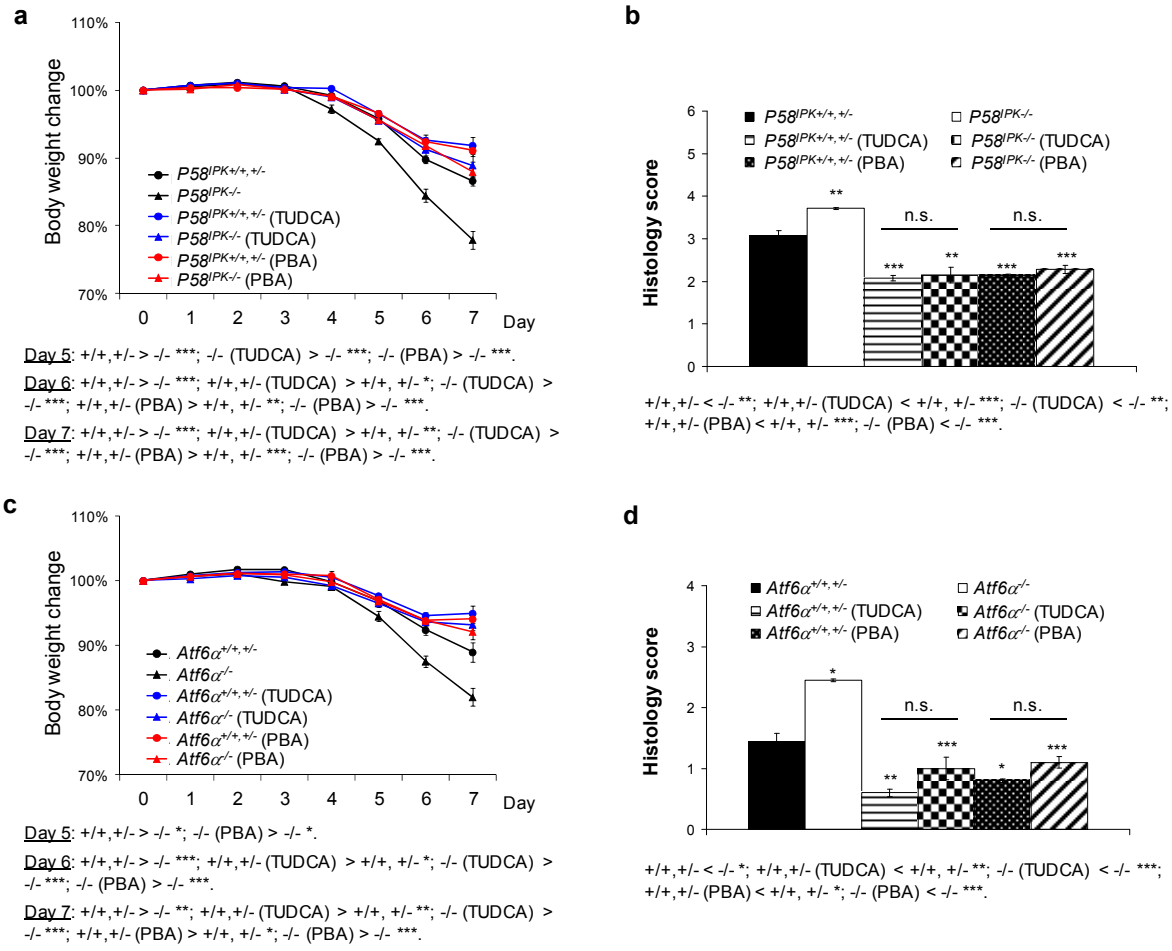

Figure 3-6: Feeding of TUDCA or PBA corrects the defects of *P58$^{IPK-/-}$* and *Atf6α$^{-/-}$* mice in response to DSS colitis. *P58$^{IPK+/+}$*, *P58$^{IPK+/-}$* and *P58$^{IPK-/-}$* mice and *Atf6α$^{+/+}$*, *Atf6α$^{+/-}$* and *Atf6α$^{-/-}$* mice were reconstituted with wild-type bone marrow cells and fed 3% DSS in drinking water for 5 days followed by 2 days of fresh water. During the same period of time, the animals received 500 mg/kg body weight TUDCA, PBA, or the same amount of PBS without TUDCA/PBA daily by gavage. Body weight **(a, c)** were measured over the 7 days. After the treatment, the colons were isolated and fixed for H&E staining and histology scoring (**b, d**). n = 7-14 for each group; *p<0.05, **p<0.01, ***p<0.001.

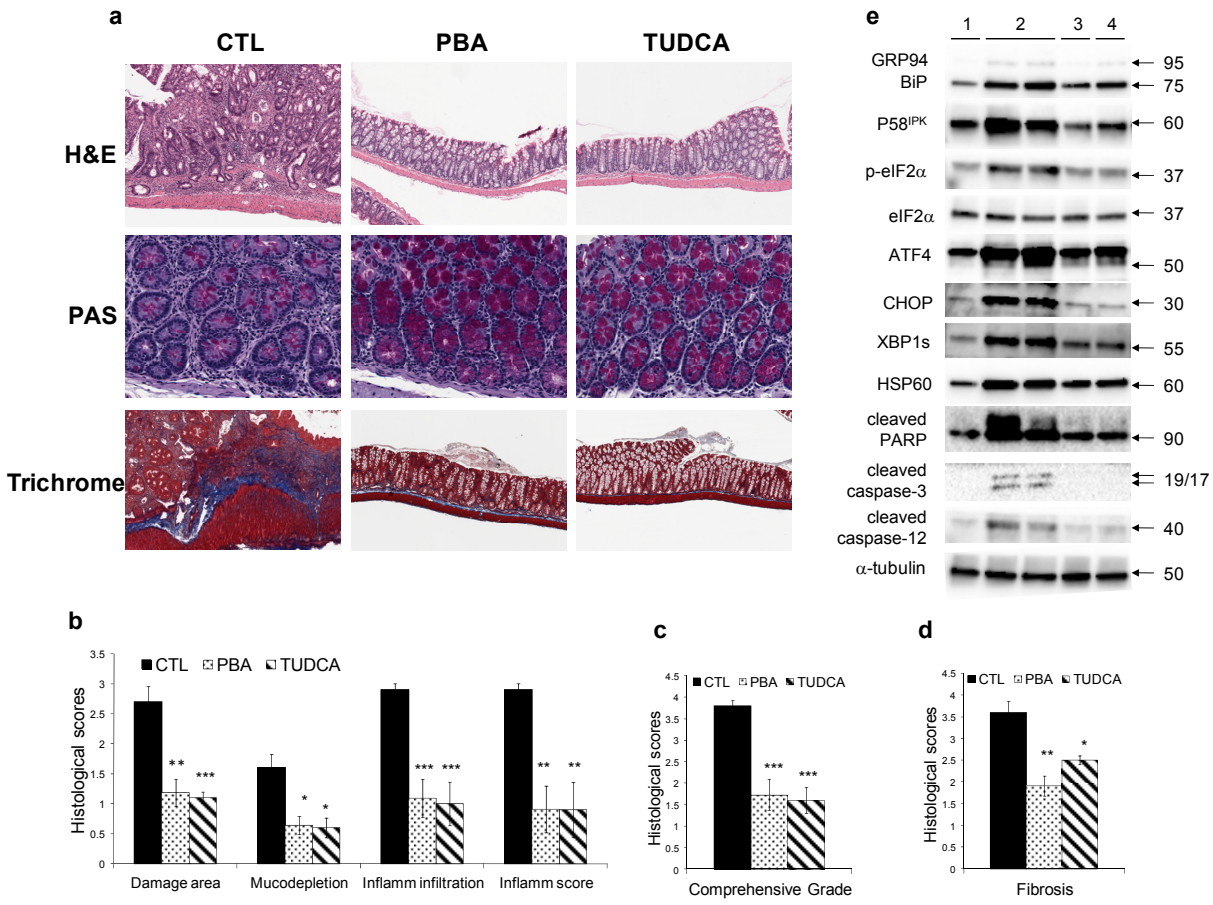

Figure 3-7: Either TUDCA or PBA alleviates chronic colitis in *Il10*$^{-/-}$ mice. *Il10*$^{-/-}$ mice with piroxicam-induced colitis received 2mg/mL PBA or TUDCA in the drinking water for 3 weeks. (**a**): Feeding of TUDCA or PBA reduces signs of chronic colitis in *Il10*$^{-/-}$ mice. Periodic-Schiff acid (PAS) staining shows mucin in goblet cells; trichrome staining indicates collagen deposition in the colon. (**b**): Histology scores are shown using the standard of Otuska et al (30). (**c**): Histology scores are shown using the standard of Berg et al (27). (**d**): Histology scores are shown for colonic fibrosis. n = 10-11 for each group. (**e**) The mice were euthanized after the experiment and the colonic IECs were isolated for protein extraction and western blotting. Representative immunoblots are shown. 1: no piroxicam-induced colitis; 2. colitis; 3. colitis→TUDCA; 4. colitis→PBA. *: $p<0.01$, **: $p<0.001$, ***: $p<0.0001$.

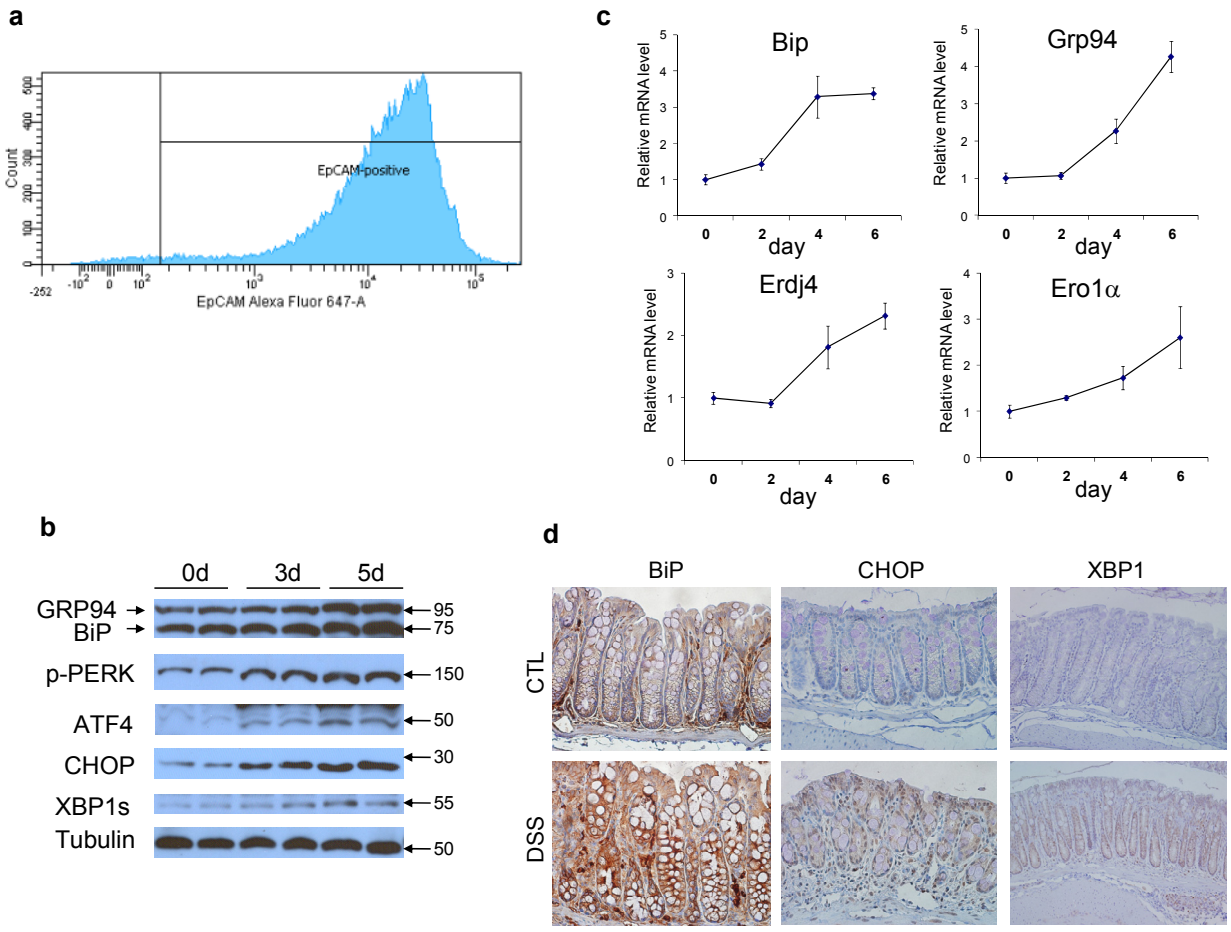

Figure 3-8: DSS colitis induces ER stress in colonic epithelial cells. (**a**) The purity of isolated colonic epithelial cells was determined by flow cytometry using an antibody against murine EpCAM. (**b**) Wild-type mice were fed 3% DSS in the drinking water for 0, 3 and 5 days. After DSS administration for the indicated days, the mice were euthanized and the colonic IECs were isolated for protein extraction and western blotting. Representative immunoblots of colon tissues from mice are shown. (**c**) Wild-type mice were fed 3% DSS in the drinking water for 0, 2, 4 and 6 days. After DSS administration for the indicated days, the mice were euthanized and the colonic IECs were isolated for RNA extraction and Q-RT-PCR. The mRNA levels were normalized to the expression of *Gapdh*. (**d**) Wild-type mice were fed 3% DSS in the drinking water for 6 days, then the mice were euthanized and the colons were removed, fixed and paraffin embedded for immunohistochemical staining of BiP, CHOP and XBP1. Representative immunohistochemical images are shown; each group contained a minimum of 6 individual mice.

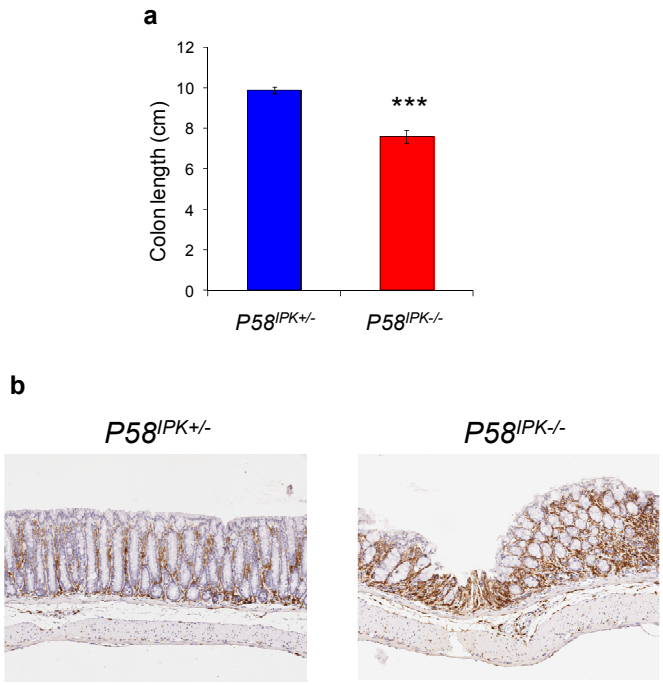

Figure 3-9: *P58*$^{IPK-/-}$ mice exhibit shorter colon lengths (**a**) and higher levels of macrophage marker F4/80 in colonic mucosa (**b**) upon DSS colitis.

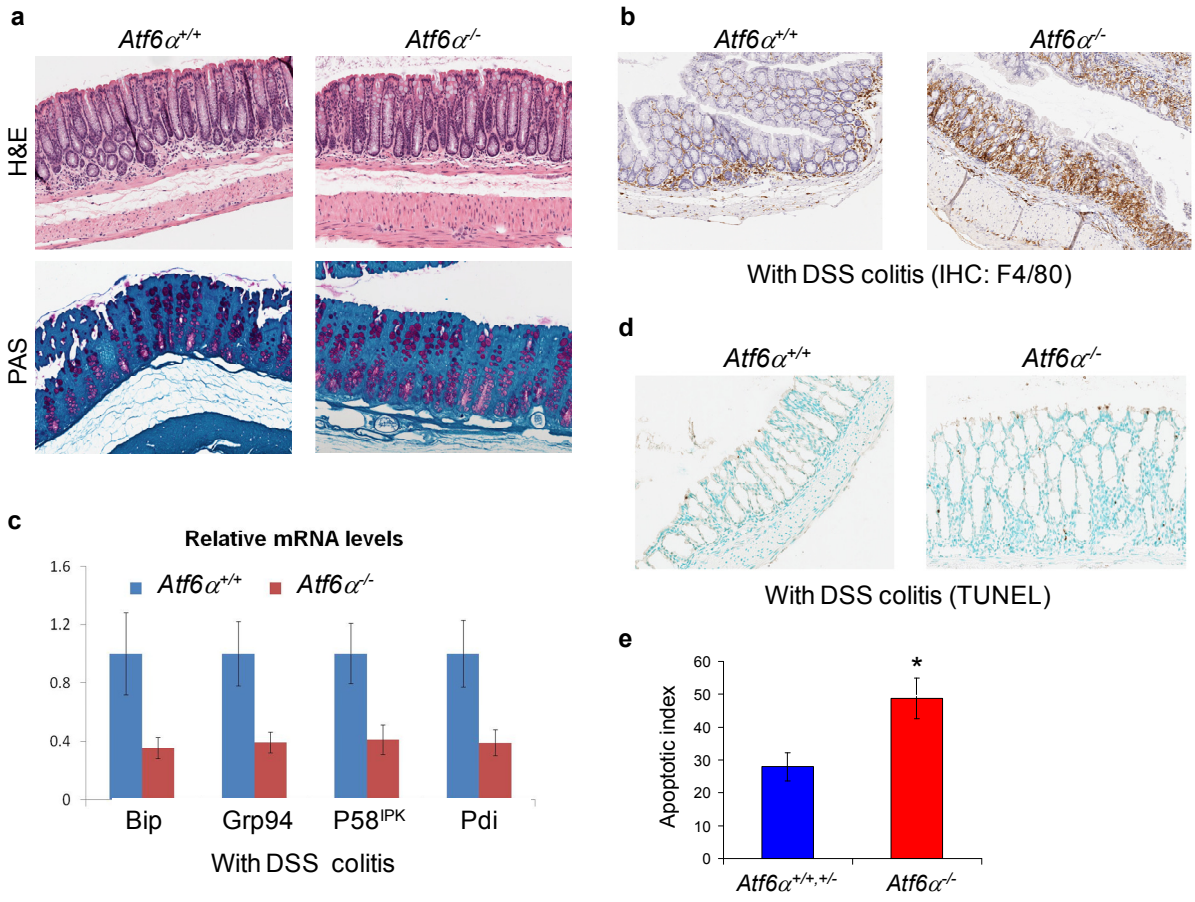

Figure 3-10: (**a**) *Atf6α*$^{-/-}$ mice display normal colon morphology under normal conditions. Colons were isolated from two-month old *Atf6α*$^{+/+}$ and *Atf6α*$^{-/-}$ littermates mice for H&E and PAS staining. (**b**) *Atf6α*$^{-/-}$ mice showed higher levels of macrophage marker F4/80 in colonic mucosa upon DSS colitis. (**c**) *Atf6α*$^{-/-}$ mice exhibit an impaired induction of ER chaperone genes including *Bip, Grp94*, *P58*^{IPK} and *Pdi* in colonic IECs upon DSS colitis. (**d**) Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)-staining of apoptotic epithelial cells in the colon sections from *Atf6α*$^{+/+}$ and *Atf6α*$^{-/-}$ mice with DSS colitis. (**e**) The apoptotic indices were calculated as the number of TUNEL-positive epithelial cells per 100 randomly selected crypts in the colon sections. 4 mice per group; * $p < 0.05$.

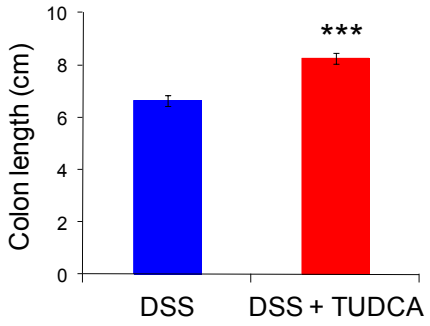

Figure 3-11: Shortening of colon lengths upon DSS colitis was significantly mitigated by the feeding of TUDCA.

Primer name	Oligo sequence (5' to 3')
rat CHOP (f)	AGAGTGGTCAGTGCGCAGC
rat CHOP (r)	CTCATTCTCCTGCTCCTTCTCC
rat BiP (f)	TGGGTACATTTGATCTGACTGGA
rat BiP (r)	CTCAAAGGTGACTTCAATCTGGG
rat 18S (f)	AGTCCCTGCCCTTTGTACACA
rat 18S (r)	GATCCGAGGGCCTC ACTAAAC
mouse IL-1beta (f)	CAACCAACAAGTGATATTCTCCATG
mouse IL-1beta (r)	GATCCACACTCTCCAGCTGCA
mouse TNF-alpha (f)	CCCTCACACTCAGATCATCTTCT
mouse TNF-alpha (r)	GCTACGACGTGGGCTACAG
mouse iNOS (f)	CAGCTGGGCTGTACAAACCTT
mouse iNOS (r)	CATTGGAAGTGAAGCGTTTCG
mouse BiP (f)	TCATCGGACGCACTTGGA
mouse BiP (r)	CAACCACCTTGAATGGCAAGA
mouse GRP94 (f)	AATAGAAAGAATGCTTCGCC
mouse GRP94 (r)	TCTTCAGGCTCTTCTTCTGG
mouse Chop (f)	GTCCCTAGCTTGGCTGACAGA
mouse Chop (r)	TGGAGAGCGAGGGCTTTG
mouse ERdj4 (f)	CCCCAGTGTCAAACTGTACCAG
mouse ERdj4 (r)	AGCGTTTCCAATTTTCCATAAATT
mouse P58IPK (f)	TCCTGGTGGACCTGCAGTACG
mouse P58IPK (r)	CTGCGAGTAATTTCTTCCCC
mouse Ero1a (f)	GCATTGAAGAAGGTGAGCAA
mouse Ero1a (r)	ATCATGCTTGGTCCACTGAA
mouse Gadd34 (f)	CCCGAGATTCCTCTAAAAGC
mouse Gadd34 (r)	CCAGACAGCAAGGAAATGG
mouse Nox2 (f)	CCCTTTGGTACAGCCAGTGAAG
mouse Nox2 (r)	CAATCCCAGCTCCCACTAACAT
mouse Bim (f)	GGAGATACGGATTGCACAGGAG
mouse Bim (r)	CCTTCTCCATACCAGACGGAAG
mouse GAPDH (f)	TTCAACGGCACAGTCAAGG
mouse GAPDH (r)	CATGGACTGTGGTCATGAG

Table 3-12: Sequence of primers used in this study.

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CHAPTER IV

EIF2α PHOSPHORYLATION IS REQUIRED FOR PANETH CELL FUNCTION AND INTESTINAL HOMEOSTASIS IN MICE

Abstract

BACKGROUND & AIMS: Recent studies link endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) to inflammatory bowel disease (IBD). Altered eIF2α phosphorylation (eIF2α-P), a regulatory hub of the UPR, was observed in mucosal tissue of patients with IBD. In this study, we examined the mechanistic role of eIF2α-P in intestinal epithelial cell (IEC) function and intestinal homeostasis in mice. METHODS: We generated mice with *villin*-Cre-mediated conditional expression of non-phosphorylatable Ser51Ala mutant eIF2α in IECs (AA^{IEC} mice). We analyzed AA^{IEC} mice under normal conditions and upon challenge with oral infection of *Salmonella* Typhimurium or dextran sulfate sodium (DSS)-induced colitis. RESULTS: Loss of eIF2α-P did not affect the normal proliferation or differentiation of IECs. However, AA^{IEC} mice expressed decreased secretory proteins including lysozyme, suggesting eIF2α-P is required for Paneth cell function. The ultrastructure of *AA* Paneth cells exhibited a reduced number of secretory granules, a fragmented ER and distended mitochondria under normal conditions. *AA* IECs displayed defective UPR signaling (e.g.

ER chaperones), mRNA recruitment to the ER and autophagy, which are crucial for the folding, maturation, and secretion of antimicrobial peptides in Paneth cells. Consequently, AA^{IEC} mice were more susceptible to oral *Salmonella* infection and DSS-induced colitis. We also show that loss of ATF4, the major downstream target of eIF2α-P, causes similar spontaneous abnormalities in Paneth cells in mice. CONCLUSIONS: eIF2α phosphorylation is required for the normal function of Paneth cells and mucosal homeostasis. This study provides the first mechanistic evidence that impaired eIF2α phosphorylation in IECs renders the intestine more susceptible to infectious and inflammatory insults.

Keywords: IBD, intestinal epithelial cells, endoplasmic reticulum stress, unfolded protein response

Introduction

In eukaryotic cells, ER is a specialized organelle responsible for the synthesis, folding, and modification of numerous secretory and membrane proteins in addition to the metabolism of carbohydrates and lipids. When cells are stimulated to secrete large amount of protein, or ER protein-folding is disrupted due to altered homeostasis, unfolded/misfolded protein accumulate in ER lumen and triggers the unfolded protein response (UPR). The UPR acts to promote ER protein-folding capacity, reduce global protein synthesis, and enhance ER associated degradation (ERAD) of misfolded protein, in order to restore ER function and improve cellular proteostasis. If UPR signaling is compromised and not able to resolve ER stress, numerous death signaling pathways are

activated [1]. In mammals, ER stress is signaled through three ER transmembrane protein sensors: inositol-requiring kinase 1 (IRE1), activating transcription factor 6 (ATF6) and PKR-like ER kinase (PERK). Activated PERK phosphorylates its substrate the α subunit of eukaryotic translation initiation factor 2 (eIF2α), which in turn inhibits the activity of the eIF2 complex and curbs translation initiation, thus decreasing global protein synthesis and reducing the ER protein load. In mammals, three cytoplasmic kinases PKR, GCN2, and HRI have been identified to phosphorylate eIF2α at the same site in response to different stress conditions. In addition to translation suppression, eIF2α-P induces selective translation of several mRNAs, including the mRNA of activating transcription factor 4 (ATF4), which transactivates the genes encoding ER chaperones including BiP, UPR-associated transcription factors, ER-Golgi translocation machinery, ERAD components, as well as amino acid synthesis and transport and the anti-oxidative stress response [2].

Mammalian intestinal epithelium contains four secretory cell types: Paneth, goblet, absorptive, and enteroendocrine cells. Paneth cells are pyramid-like columnar epithelial cells residing in the bottom of crypts in small intestine [3]. Paneth cells play a crucial role in innate immunity and host defense against bacteria, fungi and some viruses by secreting multiple antimicrobial factors including cryptdins (α-defensins), lysozyme, and phospholipase A2 [4]. As highly secretory cell types in mammals, Paneth cells are susceptible to impaired ER homeostasis and/or inefficient UPR signaling. Disrupted protein secretion function in Paneth cells is linked to Crohn's ileitis, while the diffentiation and function of colonic goblet cells are compromised in patients with ulcerative colitis [5-7]. Human genetic studies of IBD have identified primary genetic

abnormalities in several genes including *XBP1*, *AGR2*, and *ORMDL3* that encode proteins associated with ER function [8]. Previous studies suggested that the induction of ER stress through tissue-specific deletion of the gene encoding x-box binding protein 1 (XBP1) results in the absence of Paneth cells due to enhanced apoptosis, causing spontaneous inflammation in small intestine, along with an impaired host defense to bacterial infection [9]. The deletion of the gene encoding the protein disulfide isomerase AGR2, which functions in ER protein folding, causes ER stress, disrupts Paneth cell homeostasis, and disturbs mucin secretion in colonic goblet cells [10, 11].

Altered eIF2α-P has been reported in the mucosal tissues of patients with Crohn's disease or ulcerative colitis [12, 13]. However, the physiological role of eIF2α-P in IEC function and intestinal homeostasis remains elusive. In this study, we show that conditional expression of non-phosphorylatable Ser51Ala mutant eIF2α in IECs (AA^{IEC} mice) disrupts protein secretion of Paneth cells in the small intestine. *AA* IECs exhibit defective UPR signaling and autophagy, which may contribute to Paneth cell dysfucntion under normal conditions. AA^{IEC} mice are highly susceptible to oral infection of *Salmonella* Typhimurium and chemical-induced colitis, which demonstrates the physiological importance of epithelial eIF2α-P in mucosal homeostasis. In addition, mice deficient in ATF4, the major downstream target of eIF2α-P, show spontaneous abnormalities in both Paneth cells and colonic goblet cells. In this study, we demonstrate that eIF2α-P is required for the normal function of IECs; while a disregulated eIF2α-P-ATF4 pathway may contribute to human IBD.

Materials and Methods

Mice

eIF2αTgAA and *SA* transgenic mice (C57BL/6J background) were described previously [25]. All animal care and procedures were conducted according to the protocols and guidelines approved by UCUCA of the University of Michigan and IACUC of the Sanford-Burnham Medical Research Institute (SBMRI) and the University of California, San Diego.

For *Salmonella in vivo* infections, cultures of *Salmonella* Typhimurium 14028s were incubated by shaking at 37° C in LB media to late-log phase and standardized to $5x10^8$ or $1x10^9$ in HBSS media. AA^{IEC} and littermate controls were infected via oral gavage with $5x10^7$ or $1x10^8$ bacteria in 100 µl HBSS media. Animals were sacrificed at 24 hours post-infection with CO_2 followed by cervical dislocation, according to IACUC protocols. Total organs were homogenized in 5 ml sterile PBS and homogenates were diluted and pipetted onto MacConkey plates (BD Difco, Franklin Lakes, NJ) for bacterial enumeration. Sections of ileum and cecum were collected for histology and RNA analysis.

Transmission Electron Microscopy

Ileal tissues were isolated from mice and immediately immersed in 2.5% glutaraldehyde in 0.1 M Sorensen buffer, and post-fixed in 1% osmium tetroxide, and en bloc stained in 3% uranyl acetate. The tissues were dehydrated in ethanol and embedded in Epon. Ultra-thin intestinal sections were then post-stained with uranyl acetate and lead citrate. The sections were examined using a Philips CM100 electron microscope at 60

kV. Images were recorded digitally using a Kodak 1.6 Megaplus camera system operated using AMT software (Advanced Microscopy Techniques Corp.) as previously described [15].

Subcellular Fractionation of IECs

Subcellular fractions including cytosolic, ER membranous, and nuclear fractions of ileal IECs were obtained using a subcellular protein fractionation kit from Pierce (Rockford, IL) with some modifications.

DSS-induced Colitis

For acute colitis, mice received 3% (w/v) DSS (MW 36,000-50,000; MP Biomedicals, Solon, OH) in drinking water for indicated days as previously described [16].

Isolation of Intestinal Epithelial Cells

Intestinal epithelial cells were isolated as previously described [16].

Quantitative Real-Time PCR

RNAs from isolated murine colonic epithelial cells and IEC-6 cells were extracted by using RNeasy kit (Qiagen, Valencia, CA); RNA from 5 mm distal colons was isolated by using TRIzol reagent (Invitrogen, Carlsbad, CA). Q-RT-PCR was performed as previously described [16]. Q-RT-PCR results were normalized to the level of 18S rRNA or mRNA encoding glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Western Blotting

The isolated IECs were lysed in RIPA buffer for protein extraction. Protein content was measured by Protein Assay (Bio-Rad, Hercules, CA), and protein samples were analyzed by 5–20% reducing SDS/PAGE (Tris-HCl precast gels, Bio-Rad) and were detected with the following antibodies: anti-KDEL (GRP94 and BiP) (Enzo Life Sciences, Farmingdale, NY), anti-phosphor-eIF2α (Life Technologies), anti-eIF2α (Cell Signaling Technology, Danvers, MA), anti-$P58^{IPK}$ (Cell Signaling Technology), anti-ERp72 (Cell Signaling Technology), anti-SSR1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Sec11c (Abcam, Cambridge, MA), anti-β-action (Sigma-Aldrich), anti-α-tubulin (Sigma-Aldrich), anti-lysozyme (Santa Cruz Biotechnology, Santa Cruz CA), anti-LC3 (Novus Biologicals, Littleton, CO), and anti-GAPDH (Millipore Corporation, Billerica, MA). After over-night incubation of primary antibodies at 4°C, the membranes were washed and then incubated with secondary HRP-coupled antibodies (GE Healthcare, Piscataway, NJ) and developed with chemiluminescent detection system (GE Healthcare).

Histological/Immunohistochemical Staining

Periodic Schiff acid (PAS) staining and alcian blue staining (pH=2.5 or 1.0) were performed as previously published (Theory and Practice of Histotechnology, 2nd Ed. Sheehan & Hrapchak. 1987. ISBN: 0-935470-39-5).

For immunohistochemistry, formalin fixed, paraffin-embedded intestine sections were cut at 5 microns, dehydrated and pretreated with Citrate Buffer, pH 6.0 for 10 minutes. After peroxidase blocking, samples were incubated with antibody against

lysozyme (Santa Cruz Biotechnology) at a dilution of 1:200 at room temperature on the DAKO AutoStainer using the LSAB detection kit. Chromagen was applied for 5 minutes. Hematoxylin was performed for counterstaining.

Histological Scoring

Hematoxylin and eosin (H&E) stained colon sections were scored in a blinded manner by board certified veterinary pathologists as previously described by Cao et al [16].

Results

eIF2α phosphorylation is dispensible for the differentiation and proliferation of intestinal epithelial cells.

Previous studies demonstrated that homozygous Ser51Ala mutation at the phosphorylation site in eIF2α causes perinatal lethality due to defective gluconeogenesis [27]. To study the role of eIF2α phosphorylation in specific tissues, the lethality of the Ser51Ala homozyous mutation was corrected by ubiquitious expression of a LoxP-flanked wild-type *eIF2α* transgene [25]. To define the function of eIF2α-P in IECs, we crossed mice with one wild-type *eIF2α* (*S*) allele and one *eIF2α-Ser51Ala* mutant (*A*) allele, with mice expressing a floxed wild-type *eIF2α* transgene (*Tg*), and mice bearing a *villin-Cre* recombinase gene to generate *TgAA-villinCre* (AA^{IEC}) mice, which allow IEC-specific expression of non-phosphorylatable eIF2α (Figure 1). Littermate *TgSA-villinCre* and *TgAA* mice, which show no phenotypic difference, were used as controls. IECs were isolated from the small intestines and colons of two-month old AA^{IEC} and control mice to

evaluate the effect of loss of eIF2α-P in IECs. Q-RT-PCR demonstrated that the deletion efficiency of the *eIF2α Tg* was ~ 99% in the small intestine and >90% in the colon (Figure 2A). Immublotting confirmed the absence of eIF2α-P in the IECs in both small and large intestines of AA^{IEC} mice (Figure 2B).

Then, we examined whether the cell fate decisions in the ileal epithelium are altered in the absence of eIF2α-P. The expression of *Lgr5*, *Ihh* (encoding Indian Hedgehog), *Hes1*, *Math1*, and *Sox9*, which are crucial for the self-renewal and differentiation of intestinal stem cells [28], were not significantly altered in *AA* IECs by Q-RT-PCR (Figure 2C). Similarly, the expression of Paneth cell markers Cryptdin 1, 5, and 6 as well as goblet cell markers MUC 2 and 4 was not significantly different between *AA* and control IECs (Figure 2D). Periodic acid-Schiff (PAS) staining, immunostaining for chromogranin A and Ki67 staining to detect mucin-secreting goblet cells, enteroendocrine cells, and epithelial cell proliferation, respectively, in the small intestines were indistinguishable between AA^{IEC} and control mice (Figure 2E). In the epithelium of the large intestine from AA^{IEC} mice, the expression of *Lgr5*, *Sox9*, *Ihh*, *Hes1*, and *Math1* was not altered (Figure 9A). We also examined the colonic epithelia by morphology (H&E staining), epithelial cell proliferation (Ki67), neutral mucin (PAS), acidic mucin (alcian blue), and MUC2 mucin by immunohistochemistry (IHC). The colonic epithelium of AA^{IEC} mice was indistinguishable from the controls under normal conditions (Figure 9B). These data suggest that eIF2α-P is not required for the differentiation and/or proliferation of IECs in mice under normal conditions.

eIF2α phosphorylation is required for protein secretion in ileal Paneth cells.

Although the differentiation of Paneth cells was not affected in AA^{IEC} mice, PAS staining showed fewer secretory granules in the bottom of the crypts in the small intestine under normal conditions (Figure 3A). Quantification of Paneth cell granule number per crypt indicated a ~80% decrease in AA^{IEC} mice compared to controls (Figure 3B). Q-RT-PCR showed that expression of *Lysozyme* mRNA, encoding an important antimicrobial peptide in Paneth cells, was not altered in *AA* Paneth cells (Figure 3C). However, immunoblotting and IHC demonstrated the protein levels of lysozyme were dramatically reduced in *AA* Paneth cells (Figure 3D, E). Transmission electron microscopy (TEM) demonstrated *AA* Paneth cells exhibit a diminished number of secretory granules (Fig. 4), consistent with the immunoblotting, histology, and IHC data (Fig. 3). In addition, the *AA* Paneth cells displayed abnormalities in their subcellular structure under normal conditions. Where Paneth cells from control mice displayed a well-organized ER surrounding the nucleus, the ER structure was disrupted in the *AA* Paneth cells evidenced by increased vesiculation. In addition, swollen mitochondria with disrupted cristae were observed in *AA* Paneth cells (Figure 4). The ultrastructural data suggest that *AA* Paneth cells have major defects in intracellular protein trafficking with mitochondrial damage under normal conditions.

AA^{IEC} mice exhibit defective UPR activation, ER recruitment of antimicrobial mRNAs, and autophagy in ileal epithelial cells.

Given the impaired protein secretion in the *AA* Paneth cells, we examined whether the UPR was activated in these cells under normal conditions. Q-PT-PCR showed that expression of the ER chaperones/foldases *Grp94*, $P58^{IPK}$, and *Pdi*, the transcription

factors *Xbp1* and *Atf6α* was significantly reduced in ileal IECs of AA^{IEC} mice, suggesting impaired activation of the UPR (Figure 5A). IHC demonstrated the level of the ER chaperone BiP was dramatically reduced in the Paneth cells in the ileum of AA^{IEC} mice compared to the controls (Figure 10A). In addition, some components of the ER protein translocation machinery, including signal peptidase complex catalytic subunit Sec11c, signal recognition particle receptor subunit B (SRPRB), signal sequence receptor 1 (SSR1), and translocation protein Sec63, were also reduced in absence of eIF2α-P (Figure 5B). Consistently, immunoblotting demonstrated the protein levels of several ER chaperones/foldases and ER protein translocation components were reduced in *AA* IECs (Figure 5C). We next examined whether the ER-associated translation of mRNAs encoding antimicrobial peptides was altered in *AA* Paneth cells. Cytosolic and ER membranous fractions were isolated from ileal IECs, RNA extracted, and mRNA levels for the Paneth cell products lysozyme and cryptdin-1 were measured by Q-RT-PCR. The ER-associated transcripts were reduced while the transcripts in the cytoplasm were increased in AA^{IEC} mice, suggesting that ER recruitment of antimicrobial peptide mRNAs was impaired in *AA* Paneth cells (Figure 5D, E).

eIF2α-P is required for optimal induction of autophagy [29] [30, 31]. The activation of autophagy in ileal IECs, as indicated by conversion of LC3I to LC3II, was reduced in AA^{IEC} mice (Figure 5E). The expression of autophagy genes including *Atf8* and *Atg12*, previously identified transcriptional targets downstream of eIF2α-P [31-33], was reduced in *AA* IECs (Figure 5F). Autophagy is crucial for Paneth cell function in both mice and humans and several autophagy genes are associated with Crohn's ileitis [29, 31, 34-39]. Therefore, the reduced secretion of antimicrobial peptides in *AA* Paneth cells may be

explained by reduced ER protein translation/translocation, impaired UPR activation including ER protein folding components, and/or defective autophagy in absence of eIF2α-P. In spite of these abnormalities in *AA* IECs, the ER stress-associated pro-apoptotic response including *Dr5*, *Trb3*, and *Bcl2* was not altered in these cells under normal conditions (Figure 10B).

***eIF2α phosphorylation protects from oral infection by Salmonella* Typhimurium.**

As Paneth cells provide an important role in mucosal innate immunity, we tested whether AA^{IEC} mice are more susceptible to oral infection with *Salmonella* Typhimurium, which induces inflammation in ileal mucosa and penetrates the intestinal barrier to invade other organs including mesenteric lymph nodes, spleen, and liver. AA^{IEC} mice and littermate controls were infected orally with *Salmonella*. AA^{IEC} mice had significantly higher numbers of bacteria in the spleen and liver at 24 hours after infection (Figure 6A and B). *Salmonella* infection caused a stronger intestinal inflammatory response, as evidenced by induction of proinflammatory cytokines, including *Il6* and *Ccl2*, in the ileal mucosa in AA^{IEC} mice (Figure 6C). These data demonstrate the physiological importance of epithelial eIF2α-P in mucosal homeostasis and host defense in the small intestine.

eIF2α phosphorylation protects from dextran sodium sulfate (DSS)-induced colitis in the large intestine.

Since the colonic epithelium in AA^{IEC} mice displayed no morphological abnormality without stress, we challenged the mice with dextran sodium sulfate (DSS)-induced colitis. Upon DSS treatment, AA^{IEC} mice showed more severe body weight loss,

rectal bleeding, as well as epithelial damage and inflammation analyzed by histology (Figure 7A-D). Consistently, the inflammatory response was increased in the colons of AA^{IEC} mice as indicated by the induction of IFN-α and IL-1α (Figure 7E), suggesting that eIF2α phosphorylation protects the colonic epithelium from acute colitis. We then isolated colonic epithelial cells from AA^{IEC} mice and control littermates with DSS colitis to examine how UPR and apoptotic signaling were altered in the absence of eIF2α-P. Expression of ATF4, for which mRNA translation requires eIF2α-P [5 27], as well as the ATF4 ER chaperone transcriptional targets GRP94 and BiP, was compromised in *AA* colonic epithelium. In contrast, cleaved PARP, a marker of apoptotic cell death, was dramatically increased in the absence of eIF2α-P during DSS colitis (Figure 7F). These data suggest eIF2α-P is required for adaptive UPR signaling, including ATF4 and ER chaperones. In the absence of the adaptive UPR, colonic epithelial cells enter apoptosis to exacerbate DSS colitis.

Mice deficient of ATF4 exhibit spontaneous abnormalities in Paneth cell and colonic goblet cells.

Since ATF4 is an important downstream target of eIF2α-P in mammalian cells, we analyzed whether the Paneth cell phenotype observed in AA^{IEC} mice is ATF4-dependent. To test this hypothesis, we used a murine model of whole body *Atf4* deletion [40]. The ileal mucosa of $Atf4^{-/-}$ mice was morphologically normal by H&E staining (Figure 8, 1st panel), and displayed normal proliferation measured by Ki67 immunostaining (Figure 8, 2nd panel). However, IHC demonstrated reduced lysozyme in the ileum of $Atf4^{-/-}$ mice compared with $Atf4^{+/+,+/-}$ littermates, suggesting a spontaneous Paneth cell defect

in the ileum (Figure 8, 3rd panel). *Atf4*$^{-/-}$ mice exhibit normal enteroendocrine cells (Figure 8, 4th panel). Given the similar Paneth cell phenotypes we observed in the *AA*^{IEC} and *Atf4*$^{-/-}$ mice, it is plausible that eIF2α-P supports Paneth cell function through its downstream target ATF4.

While *AA*^{IEC} mice show no abnormality in their large intestine under normal conditions, *Atf4*$^{-/-}$ mice exhibit spontaneous dysfunction in colonic goblet cells by histology. PAS (neutral mucin) and alcian blue (pH=2.5, acidic mucin) double staining showed reduced alcian blue-positive staining on the top half of the colonic mucosa in *Atf4*$^{-/-}$ mice (Figure 11, upper panel). Alcian blue (pH=1.0, sulfated mucin) further indicated decreased sulfated mucin in *Atf4*$^{-/-}$ mice [28] (Figure 11, second panel). These data suggest colonic goblet cells in *Atf4*$^{-/-}$ mice have major defects in the synthesis of acidic and sulfated mucins, which are important for intestinal homeostasis and host defense [28-31]. We also stained the colon sections with an antibody that specifically recognizes ER-localized MUC2 mucin [32, 33]. The immunohistochemistry demonstrated high level of ER-localized MUC2 mucin in the colons of *Atf4*$^{-/-}$ mice, which suggests that the modification of MUC2 mucin in the ER and/or ER-Golgi trafficking are impaired in absence of ATF4 (Figure 11, lower panel).

Discussions

The physiological importance of eIF2α-P was highlighted by previous studies. Mice with homozygous knock-in Ser51Ala mutation at eIF2α phosphorylation site were perinatal lethal due to severe hypoglycemia associated with defective liver gluconeogenesis [26]. Later, eIF2α-P was shown to be essential for optimal expression of

many UPR genes and the maintenance of cellular homeostasis and normal function in multiple cell types, including fibroblasts, osteoblasts, hepatocytes, as well as pancreatic endocrine and exocrine cells [25, 26, 41, 42]. In IBD patients, eIF2α-P was increased in mucosal tissue with active disease [23]. However, recent studies showed that eIF2α-P was suppressed in unaffected mucosal tissue of patients with ulcerative colitis [24]. In this study, we investigated the physiological importance of eIF2α-P in IECs by analysis of mice with tissue specific ablation of eIF2α-P in IECs. While the proliferation and differentiation of IECs in both small and large intestines of AA^{IEC} mice was not altered, Paneth cells displayed major defects in the production and secretion of antimicrobial peptides, including lysozyme. Ultrastructural analysis revealed altered intracelluler structures in *AA* Paneth cells including diminished secretory granules, ER fragmentation and damaged mitochondria, suggesting defective protein folding, maturation and secretion, as well as oxidative stress. Biochemical analyses demonstrated *AA* IECs are defective in UPR signaling, ER-associated mRNA translation and possibly autophagy. These three machineries combined together may lead to the compromised protein secretion observed *AA* Paneth cells. Defective recruitment of mRNAs to the ER reduces the level of antimicrobial peptides that enter the ER. It was unexpected that Ser51Ala mutant eIF2α results in a translation defect. We propose that initial removal of eIF2α phosphorylation increases protein synthesis to induce ER stress. In the absence of ATF4 mRNA translation, the defective adaptive UPR may cause the complete collapse of the secretory pathway. It is interesting that defects in the IRE1/XBP1 branch decrease expression of Paneth-cell specific mRNAs[13], whereas inactivation of the PERK/eIF2α branch does not

affect Paneth cell differentiation or survival, but causes a post-transcriptional block in synthesis of antimicrobial peptides.

Recent animal and human studies link autophagic defects to Paneth cell dysfunction and Crohn's ileitis, suggesting that autophagy is required for intracellular trafficking and secretion of antimicrobial peptides in Paneth cells [43]. Interestingly, the phenotype of *AA* Paneth cells including diminished secretory granuels, increased intracellular vesicles, and swollen mitochondria is reminiscent to the subcellular features of Paneth cells with defective autophagy [39], which suggests the physiological relevance of eIF2α-P-induced autophagy in Paneth cell function. eIF2α-P plays an important role in autophagic induction in response to different stresses in cultured cells [29, 31, 36-38]. In addition to the role of eIF2α-P on a cellular level, we also demonstrate the physiological significance of this signaling pathway in intestinal homeostasis by showing that AA^{IEC} mice are more susceptible to enterobacterial infection, as indicated by higher level of mucosal inflammation and systemic infection upon *Salmonella* invasion.

Although the large intestine of AA^{IEC} mice is morphologically indistinguishable, these mice are more sensitive to DSS-induced colitis, as demonstrated by higher clinical and histological scores and epithelial cell death. The colonic IECs from AA^{IEC} mice showed impaired induction of ATF4 and ER chaperones, including GRP94 and BiP, upon DSS colitis. Recently, we showed that the ER chaperone response protects against apoptosis of colonocytes and DSS colitis [22]. Therefore, eIF2α-P may improve the cellular homeostasis of colonic IECs through induction of the adaptive UPR, including ER chaperones. Given the protective role of eIF2α-P in IEC function and survival in small and large intestines, future studies should determine whether small molecular regulators

of eIF2α-P signaling, such as the inhibitors of eIF2α dephosphorylation salubrinal or guanabenz [44-46], could alleviate signs of IBD in animal models.

In addition to PERK, there are three cytosolic eIF2α kinases identified in mammals: dsRNA-activated protein kinase (PKR), general control nonrepressed 2 (GCN2), and heme-regulated eIF2α kinase (HRI). PKR is activated by dsRNA as well as inflammatory signals including LPS and TNFα, and ER stress, which are physiologically relevant stimuli in inflamed intestine [47-49]. In a previous study, we demonstrated that PKR contributes to eIF2α-P in colonic epithelium, which protects against DSS-induced colitis in mice [20]. Given that the deletion of *Pkr* did not abolish eIF2α-P completely, PERK and/or another cytosolic kinase may also play a role in the phosphorylation of eIF2α in colonic IECs. Studies using conditional deletion of these kinases will provide insight into how eIF2α-P is regulated in IECs upon the challenge of inflammatory insults.

Previous studies showed that loss of the ER stress-induced transcription factor XBP1 in intestinal epithelial compartment causes progressive Paneth cell death and spontaneous enteritis in ~60% mice [13]. In our study, AA^{IEC} mice did not exhibit increased Paneth/goblet cell death in spite of disrupted Paneth cell function and higher sensitivity to enterobacterial infection and experimental colitis. In addition, we did not observe any spontaneous inflammation in the gut of AA^{IEC} mice when housed at the animal facility of SBMRI, which is different from the phenotype of the $Xbp1^{flox/flox}VillinCre$ mice [13]. This could be explained by 1) the small intestine of AA^{IEC} mice have Paneth cells present, which serve as an important niche for intestinal stem cells and Paneth cells are probably important for IEC renewal [26]; 2) the *AA* Paneth cells have residual function including secretion of antimicrobial peptides; and/or 3) housing facility may impact the

development of enterocolitis in mice. In contrast to XBP1 and eIF2α-P, ATF6α and ATF6β seem dispensable for Paneth cell function in mice (our unpublished observations), suggesting the roles of the three UPR branches are different in the function and/or survival of Paneth cells in mice. Finally, the phenotype of *Atf4*$^{-/-}$ mice in the small intestine suggests that the Paneth cell defects in *AA*^{IEC} mice are ATF4-dependent. *Atf4*-whole body knockout mice have defects in multiple organ systems [40, 50, 51] and are extremely difficult to breed. Future studies will require analysis of ATF4 in Paneth cell function using *Atf4* conditional knockout mice.

Acknowledgments

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S: wt *eIF2α*; ***A***: *eIF2α-Ser51Ala*; ***Tg***: floxed wt *eIF2α* transgene

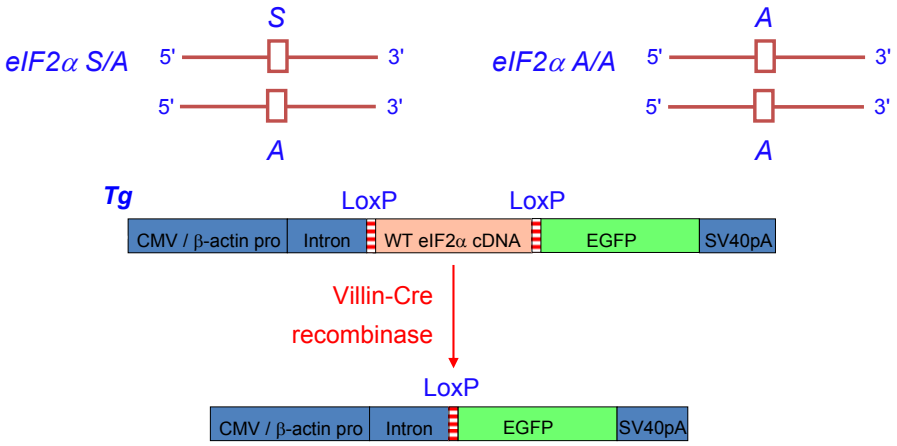

Figure 1: Generation of AA^{IEC} and control mice.

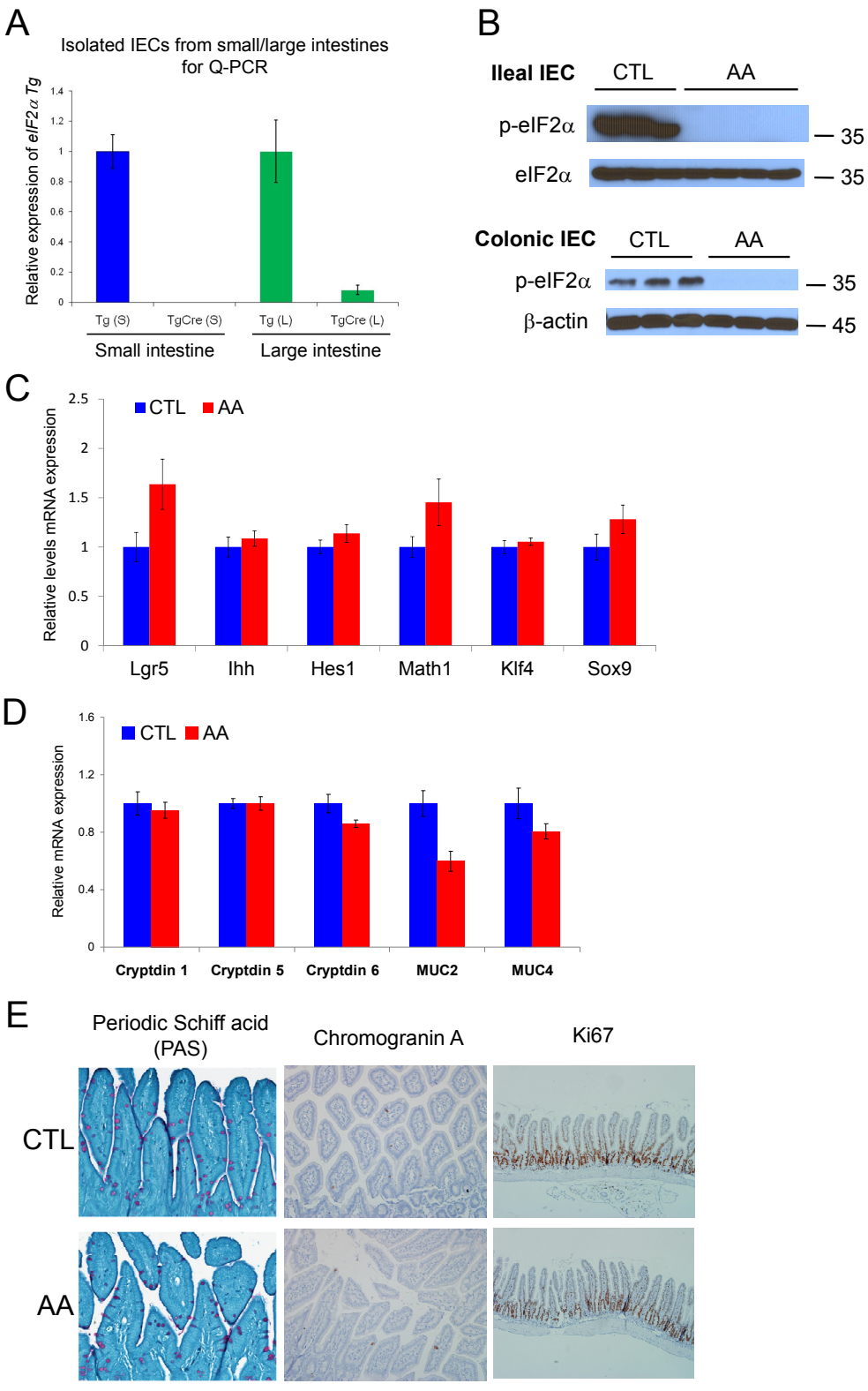

Figure 2: eIF2α phosphorylation does not affect the proliferation or differentiation of IECs. A: Deletion of eIF2α transgene (*Tg*) in IECs of small and large intestines in

AA^{IEC} mice. B: Analysis of eIF2α-P in IECs of small and large intestines in AA^{IEC} mice by immunoblotting. C and D: Gene expression in IECs from the ileums of AA^{IEC} and control mice. No significant difference was found between *AA* and control groups. E: IHC analysis of ileums from AA^{IEC} and control mice. Ileums were isolated, fixed, and embedded for histological and IHC staining for markers of goblet cells, enteroendocrine cells, and IEC proliferatio. Representative pictures are shown. N=4-8 for each group.

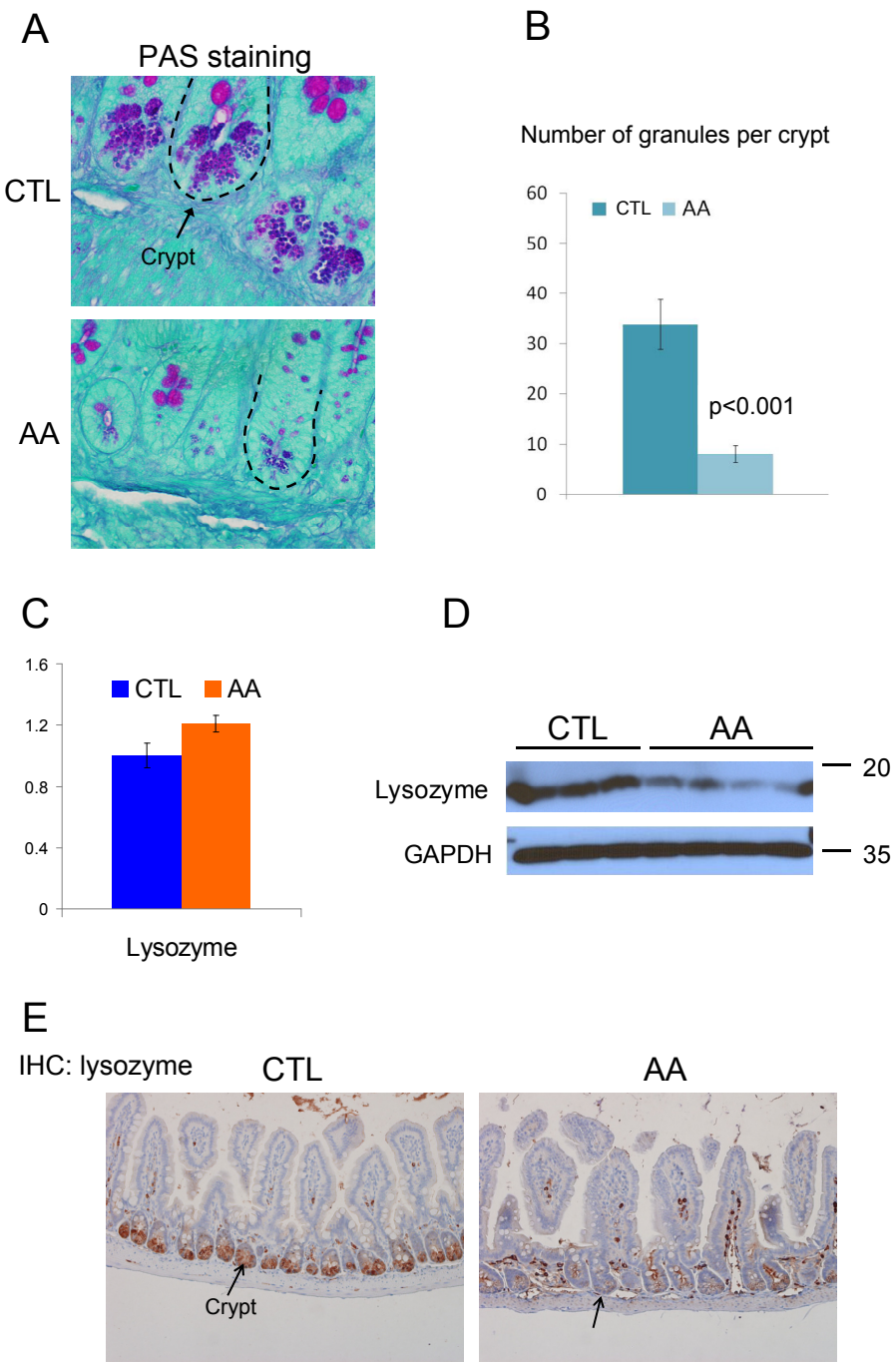

Figure 3: eIF2α phosphorylation is required to maintain protein secretion in ileal Paneth cells under normal conditions. A: The ileums of AA^{IEC} and control mice analyzed by PAS staining. The dash lines denote single crypts in the ileums. B: Number of secretory granules quantified based on the PAS staining. C-E: Analysis of Ileal IECs from control and AA^{IEC} mice. Ileums were isolated from AA^{IEC} and control mice for RNA, protein, and immunohistochemical analyses. N=5-11 for each group.

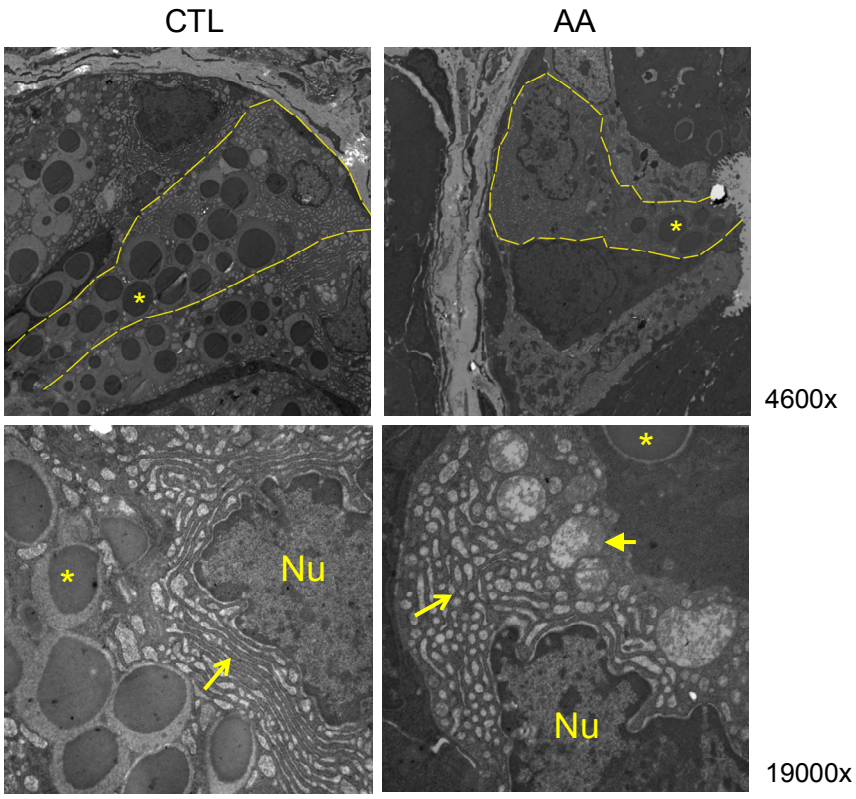

Figure 4: eIF2α phosphorylation is required to preserve the secretory pathway and secretory granule production under normal conditions. The ileums of AA^{IEC} and control mice were isolated, fixed, and stained for TEM analysis. Representative pictures are shown.

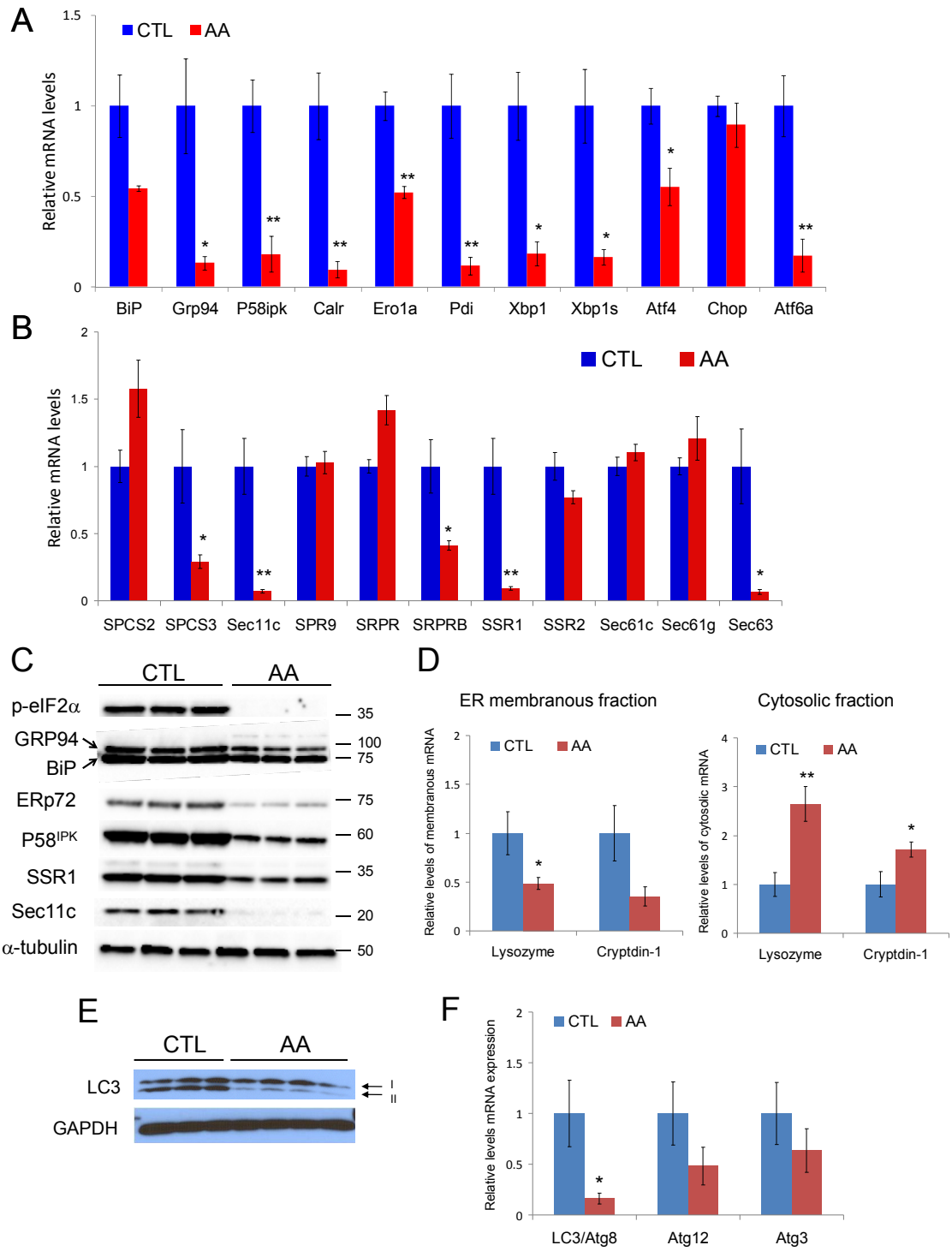

Figure 5: eIF2α phosphorylation is required for UPR activation, ER protein co-translational translocation, and autophagy under normal conditions. Ileal IECs were isolated from the ileums of AA^{IEC} and littermate control mice for RNA and protein analyses. A: The levels of UPR components detected by Q-RT-PCR. B: The levels of ER

protein translocation machinery components detected by Q-RT-PCR. C: Immunoblotting of UPR proteins and ER protein translocation components. D: The levels of mRNAs encoding antimicrobial peptide in ER membranous and cytosolic fractions isolated from ileal IECs. E: Conversion of LC3 in isolated ileal IECs detected by immunoblotting. F: The levels of autophagy components in ileal IECs detected by Q-RT-PCR. N=6-8 for each group. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

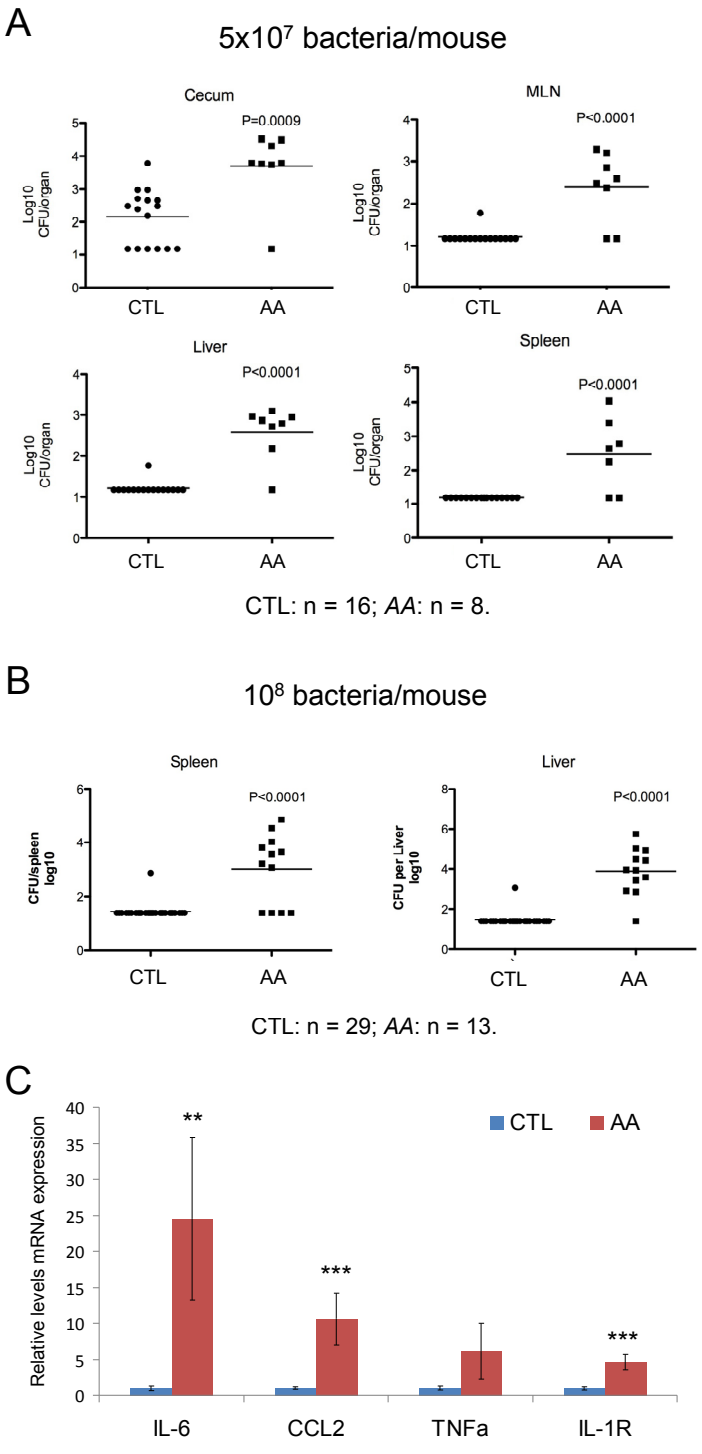

Figure 6: eIF2α phosphorylation is required to protect from oral infection with *Salmonella*. AA^{IEC} and control mice were given $5x10^7$ (A) or 10^8 (B, C) of *Salmonella* by gavage. After 24 hours, the mice were euthanized and organs harvested for RNA extraction (C, ileal tissue) or bacterial culture and counting of colony numbers (A, B). N=8-29 for each group. *: p<0.05, **: p<0.01, ***: p<0.001.

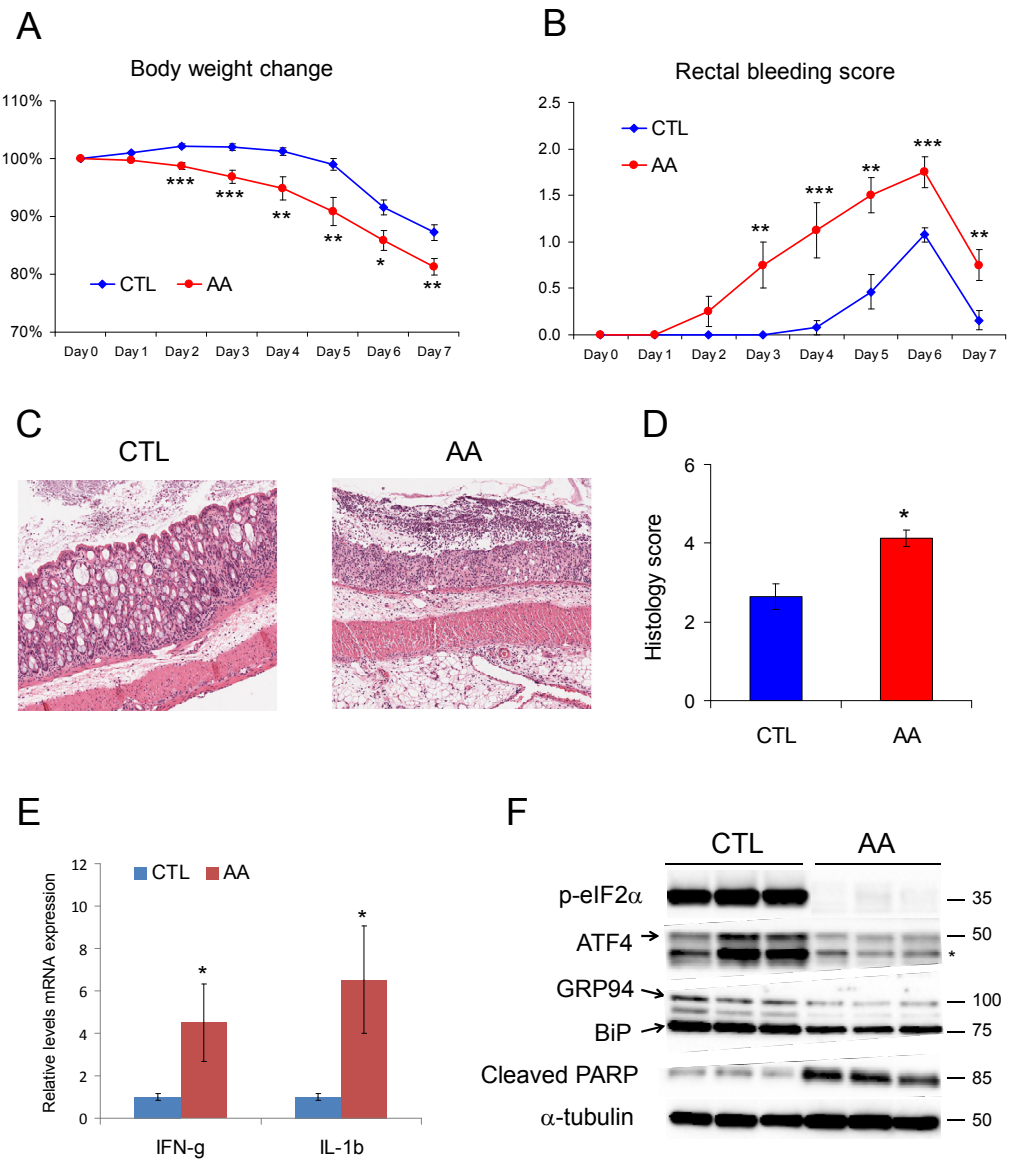

Figure 7: eIF2α phosphorylation protects from dextran sodium sulfate (DSS)-induced coltis. AA^{IEC} and control mice were challenged by 3% DSS in drinking water for 5 days followed by 3 days of fresh water. A: Body weight loss. B: Rectal bleeding. C: H&E staining after DSS challenge. AA^{IEC} and control mice were euthanized and colons harvested for H&E staining. D: Histology scores of DSS colitis. E: Inflammatory mediators in colonic tissue after induction of DSS colitis measured by Q-RT-PCR. E: The activation of UPR and apoptotic signaling in colonic IECs after induction of DSS colitis by immunoblotting. N=8-11 for each group. *: p<0.05, **: p<0.01, ***: p<0.001.

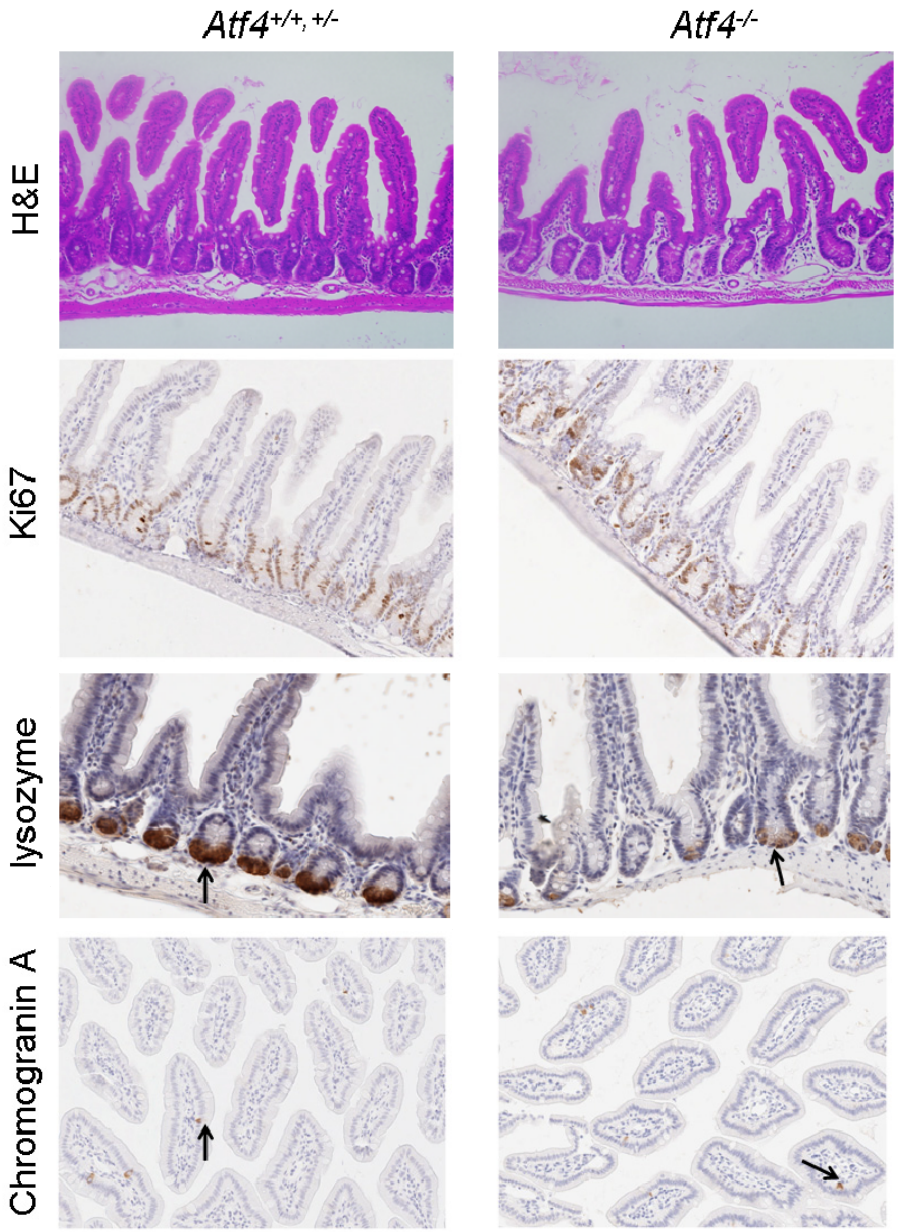

Figure 8: Histological and IHC staining of ileal sections from *Atf4*$^{+/+,+/-}$ and *Atf4*$^{-/-}$ littermate mice under normal conditions. Representative pictures are shown.

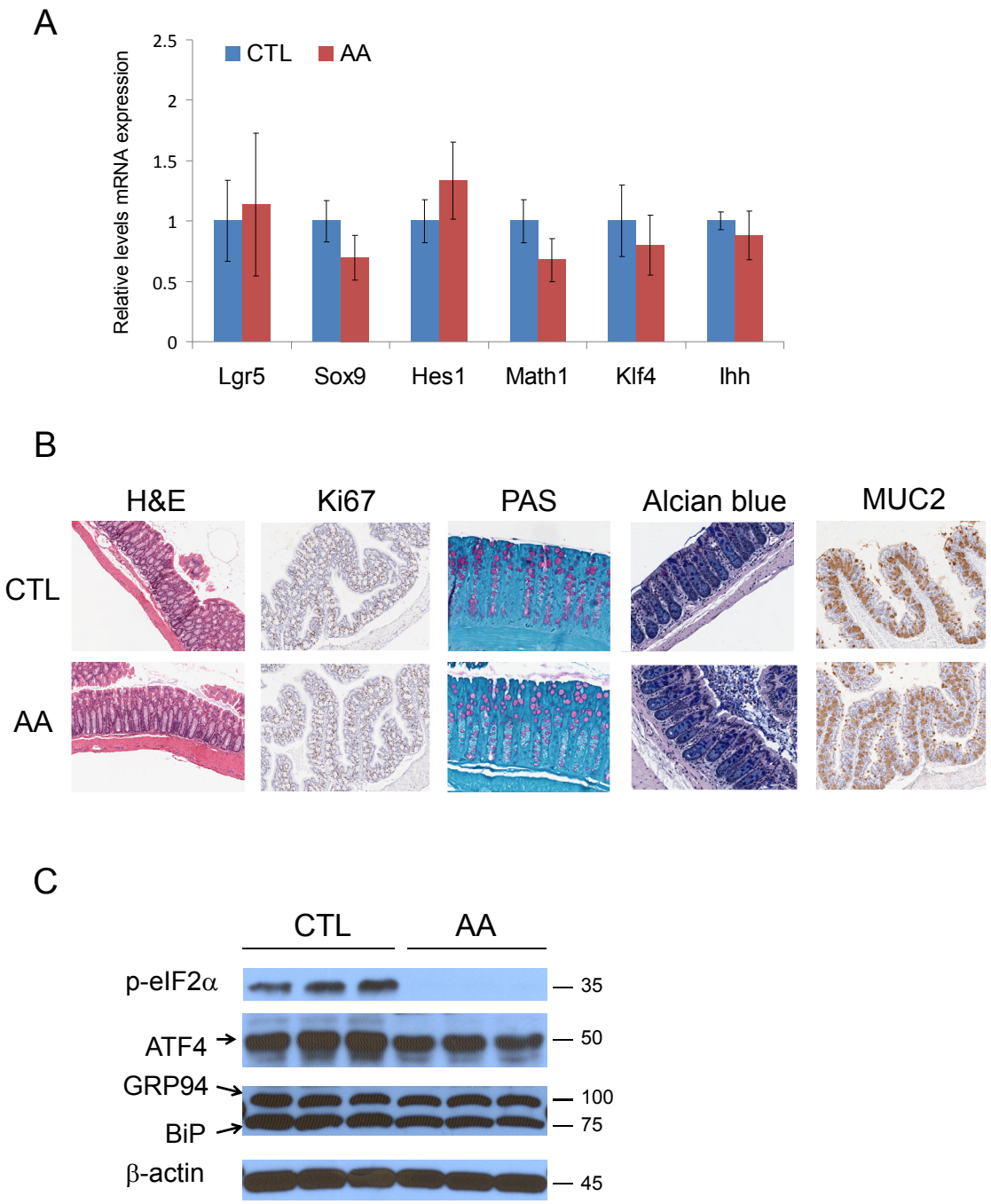

Figure 9: The phenotype of colonic epithelium in AA^{IEC} mice under normal conditions. A: IECs were isolated from the colons of AA^{IEC} and control mice for RNA extraction, cDNA synthesis, and Q-RT-PCR. No significant difference was found between *AA* and control groups. B: Histological and immunohistochemical staining of colon sections from CTL and *AA* mice without any stress. Representative pictures are shown. E: The activation of UPR signaling in colonic IECs under normal conditions by immunoblotting.

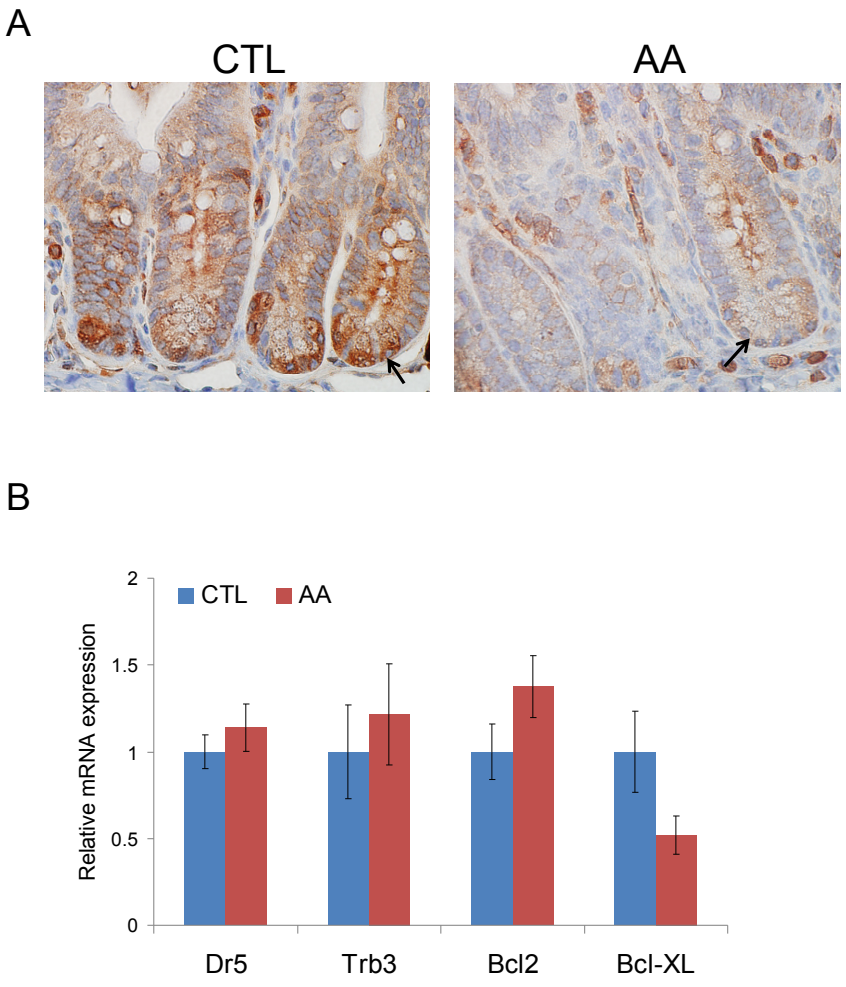

Figure 10: The phenotype of ileal epithelium in *AA*^{IEC} mice under normal conditions. A: immunostaining of BiP on ileal sections from CTL and *AA* mice, representative crypts are shown. B: The activation of ER stress-associated pro-apoptotic signaling in ileal IECs under normal conditions by Q-RT-PCR.

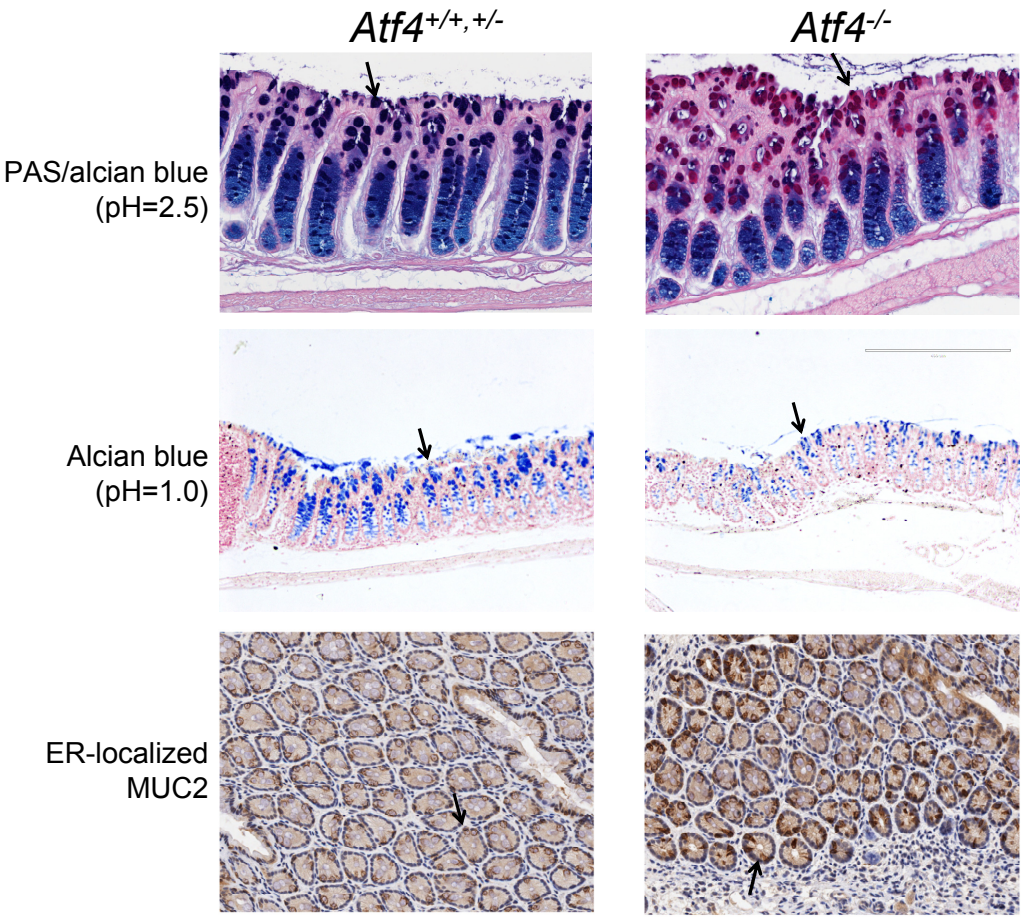

Figure 11: Histological and immunohistochemical staining of colon sections from *Atf4*$^{+/+,+/-}$ and *Atf4*$^{-/-}$ litermate mice. PAS, alcian blue (pH = 2.5 or 1.0) staining and immunohistochemistry using an antibody that specifically recognizes ER-localized MUC2 mucin.

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CHAPTER V

CONCLUSIONS, PERSPECTIVES, AND FUTURE DIRECTIONS

The role of ER stress and the UPR in intestinal inflammation was first implicated in a study by Bertolotti et al., which showed that deletion of *Ire1β* exacerbated experimental colitis in mice [1]. However, the first landmark finding in this new field had not been made until 2008, when some hypomorphic alleles of *XBP1* were identified to associate with both human ulcerative colitis and Crohn's disease [2]. In the same study, Kaser et al. showed that loss of XBP1 in the intestinal epithelial compartment was sufficient to induce spontaneous enteritis in mice, probably by causing Paneth cell death, goblet cell dysfunction, and hyperactivated inflammation in the epithelium. As a first-year PhD student, I was inspired by these findings and decided to further our understanding of how the UPR orchestrates IEC function and mucosal homeostasis during IBD pathogenesis.

EIF2α-ATF4 pathway: another UPR branch essential for intestinal epithelial function and mucosal homeostasis

I first studied the role of PKR, a cytosolic kinase of eIF2α, in IEC function and DSS-induced colitis. In addition to dsRNA, PKR can be activated by ER stress as well as

a number of inflammatory signals, e.g. LPS and TNF-α, which are induced in inflamed intestine [3, 4]. My data showed that PKR in the non-hematopoietic compartment, but not the hematopoietic compartment, protects against DSS-induced colitis. The protection is conferred by eIF2α phosphorylation-dependent UPR activation including ER chaperone genes, as well as prosurvival signaling p-STAT3 and p-AKT in colonic IECs during colitis. The phenotype I observed in *Pkr*$^{-/-}$ mice led me to directly examine the function of eIF2α phosphorylation in IECs. As expected, a large number of UPR components including ER foldases and transcription factors were downregulated in ileal IECs of *AA*^{IEC} mice under normal conditions. In addition, the ER-associated protein translation/translocation and autophagic activation (discussed below) are also impaired in ileal IECs in *AA*^{IEC} mice. The three mechanisms combined together may cause the protein secretion defects observed in *AA* Paneth cells, which increased the susceptibility to oral infection of *Salmonella*. Now we are not able to distinguish whether all three mechanisms contribute significantly to the Paneth cell defects in *AA*^{IEC} mice, or one or two of them play a predominant role. One way to dissect this question is to treat *AA*^{IEC} mice with chemical chaperones that boost ER protein folding homeostasis, or carbamazepine, an autophagy activator, to determine whether these treatments can rescue the Paneth cell phenotype. To study how loss of eIF2α phosphorylation in IECs affects mucosal inflammation in the small intestine, we could cross *AA*^{IEC} mice with *Tnf*$^{\Delta ARE}$ mice, a spontaneous model of Crohn's ileitis in the future. Similar to what we observed in *Pkr*$^{-/-}$ mice, *AA*^{IEC} mice are more vulnerable to DSS-induced acute/chronic colitis. Future studies should determine how eIF2α phosphorylation regulates the maturation and secretion of mucins in colonic goblet cells.

Autophagy is an essential catabolic process in both unicellular eukaryotes and metazoans. In addition to its role in regulating nutrient availability in starved cells, autophagy selectively removes aggregated/misfolded proteins and damaged organelles, and clear pathogens and apoptotic bodies in immune/autoimmune responses [5-10]. Recent studies showed that autophagy plays important and diverse roles in intestinal homeostasis by orchestrating protein trafficking and secretion in Paneth cells, clearance of intracellular bacteria (xenophagy), and cytokine secretion. GWAS or candidate gene approaches have identified a number of autophagic alleles associated with Crohn's disease, including *ATG16L1*, *NOD2*, *IRGM*, *LRRK2-MUC19*, *ULK1*, *ATG2A*, and *GABARAPL1* [7]. DAP1, a novel substrate of mTOR and negative regulator of autophagy, was recently implicated in ulcerative colitis [11, 12]. Variants within the gene encoding protein tyrosine phosphatase nonreceptor type 2 (PTPN2), which regulates autophagosome formation in IECs, are associated with both Crohn's disease and ulcerative colitis [13-15]. These studies have started to challenge our previous understanding that autophagy is exclusively associated with Crohn's disease but not ulcerative colitis [16, 17]. However, it remains unknown how defective autophagy contributes to ulcerative colitis. Given the critical role of autophagy in Paneth cells, it will be worthwhile to determine how this signaling regulates the function of goblet cells, another secretory cell type in the gut. Studies in the past decade linked ER stress to autophagy by showing that the three UPR branches regulate autophagy at different stages including induction, vesicle nucleation, elongation, and maturation [18]. Meanwhile, autophagy also impacts ER protein folding homeostasis and the activation of UPR [19-21](. Given that both autophagy and ER stress/UPR are crucial in IBD-related signaling

including protein secretion, energy balance, and inflammatory responses, future studies should determine how autophagy and ER stress interact in IEC function and mucosal homeostasis.

In addition to AA^{IEC} mice, we also studied the mice deficient in ATF4, for which mRNA translation requires eIF2α phosphorylation. *Atf4*$^{-/-}$ mice displayed an interesting phenotype in both small and large intestines. Similarly to AA^{IEC} mice, *Atf4*$^{-/-}$ mice exhibited Paneth cell defects in the ileum under normal conditions. In addition, *Atf4*$^{-/-}$ mice showed a spontaneous abnormality in goblet cell function in the colon, including reduced production of acidic mucins and increased accumulation of MUC2 precursor in the ER. These findings suggest that *Atf4*$^{-/-}$ goblet cells have major defects in post-translational modification (i.e. *N*-/*O*-glycosylation) and/or intracellular trafficking of mucins. RNA-seq analysis of murine embryonic fibroblasts (MEF) deleted in *Atf4* found four transcripts the were dramatically reduced in *Atf4*$^{-/-}$ MEFs: β1,4-N-acetylgalactosaminyltransferase III (*B4galnt3*), UDP-N-acetyl-α-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 1 (*Galntl1*), UDP-N-acetyl-α-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 3 (*Galntl3*), UDP-N-acetyl-α-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 4 (*Galntl4*). GalNAc-transferases (GALNTs) orchestrate the initiation of mucin-type *O*-linked protein glycosylation by transferring *N*-acetylgalactosamine to serine/threonine amino acid residues [22]. B4GALNT3, an enzyme highly expressed in the gastrointestinal tract, transfers GalNAc to nonreducing terminal GlcNAc-β in the synthesis of N,N'-diacetyllactosediamine and GalNAcb1,4GlcNAc [23,

[24]. These enzymes may be important for modification, folding and/or trafficking of MUC2 mucin in colonic goblet cells [25].

In the future, studies should study the effect of knockdown of *Atf4* or its downstream targets on mucin maturation and secretion in mucin secreting HT29-MTX/FU cells or goblet-like LS174T cells *in vitro*. Given the difficulty to breed *Atf4*-whole body knockout mice at SBMRI, *Atf4* conditional knockout mice (*Atf4*$^{fe/fe}$-*VillinCre*) could be used to explore the role of ATF4 in Paneth and goblet cell function *in vivo* [26].

From molecular chaperones to chemical chaperones: therapeutic implications from basic science findings

Induction of ER chaperones and foldases is an important strategy cells employ to cope with protein misfolding in the ER. Since the ER chaperone response is critical for protein folding homeostasis *in vitro* and *in vivo*, I investigated how UPR signaling can chaperone gene expression impact IEC function and mucosal inflammation in mice. P58^{IPK}, an ER co-chaperone for BiP, and ATF6α, the master activator of ER chaperone genes in mammals, protect mice against DSS-induced colitis. In mice with defective ER chaperone induction, the apoptotic UPR was activated to increase IEC death upon DSS challenge. Interestingly, we found that feeding mice with chemical chaperones rescued the genetic deletion of *P58*^{IPK} and *Atf6α* during the progression of DSS-induced colitis, supporting the notion that the chemical chaperones function in a similar manner as ER molecular chaperones.

The findings that ER chaperone response improves colitis inspired me to explore the therapeutic potential of chemical chaperones in IBD. The chemical chaperones TUDCA and PBA suppressed ER stress in cultured IECs treated with inflammatory stimuli. Feeding of either TUDCA or PBA mitigated signs of colitis in three murine models of colitis by reducing ER stress in the IECs. At this point it is unknown whether chemical chaperones act on IECs alone or other cell types are also affected in the intestine. Future studies should determine how TUDCA and PBA affect the differentiation, activation, and migration of immune cells during the pathogenesis of IBD in murine models.

TUDCA and PBA are FDA-approved drugs that have outstanding safety profiles in humans. We are trying to identify collaborators for a Phase I clinical trial of the two compounds. Current IBD therapies including steroids, immunosuppressants and biologics heavily rely on intervention of the inflammatory response and have significant risks and side effects. If the efficacy and safety of TUDCA and PBA can be demonstrated in IBD patients, they could fill a needed gap in current IBD therapies.

Ongoing studies on ER stress in IBD pathogenesis and therapeutics

Although eIF2α-phosphorylation is important for IEC function, a previous study showed that increased eIF2α-phosphorylation is associated with reduced heat shock protein synthesis in the mucosa of active IBD patients and *Il10*$^{-/-}$ mice with colitis [27]. Moreover, sustained eIF2α-phosphorylation induces CHOP that aggravates DSS colitis [28]. To elucidate the functional impact of uncontrolled eIF2α-phosphorylation on intestinal inflammation, we are studying mice with deletion of *Gadd34* [29], encoding the regulatory

subunit of protein phosphatase 1 that targets eIF2α dephosphorylation. We will test the hypothesis that sustained eIF2α-phosphorylation in IECs of *Gadd34*$^{-/-}$ mice will shut down synthesis of HSPs, pro-survival factors, mucins, and antimicrobial peptides that are essential for cellular and mucosal homeostasis, and promote the pro-apoptotic signaling downstream of CHOP.

For the chemical chaperone project, I am currently testing the efficacy of TUDCA and PBA in Crohn's ileitis models of *Tnf*$^{\Delta ARE}$ and SAMP1/YitFcsJ mice. Our success in the chemical chaperone studies motivated me to examine other small molecules that regulate the UPR in IBD models. Given the importance of eIF2α phosphorylation in IEC function and mucosal homeostasis, I am testing two inhibitors of eIF2α dephosphorylation guanabenz and salubrinal on chronic DSS colitis, *Il10*$^{-/-}$ and *Tnf*$^{\Delta ARE}$ models [30, 31]. The IRE1-XBP1 pathway plays crucial roles in the differentiation and/or activation of several immune cells including T lymphocytes, plasma cells, dendritic cells, and macrophages, all of which profoundly impact IBD pathogenesis [32-35]. I am examining the efficacy of an IRE1α RNase inhibitor (MKC4485) in *Il10*$^{-/-}$ and *Tnf*$^{\Delta ARE}$ mice and in mucosal explants from these two mice. In addition, I am screening novel bile acid derivatives for chemical chaperone activity using goblet-like LS174T cells and non-transformed IEC-6 cells. In the future, we hope to study differentiation of intestinal stem cells and epithelial organoids for *in vitro* pharmacological studies.

When I communicate with biomedical researchers, especially people outside the ER stress field, the question they ask most frequently is how to detect "protein misfolding" in IECs during IBD. Greater than 95% of publications on ER stress measure UPR activation by Q-PCR, immunoblotting, and/or immunostaining as a sole indicator of

ER stress, i.e. protein misfolding in the ER. These are relatively easy techniques but are limited because their activation may not require protein misfolding in the ER. First, as a non-ligand specific process, UPR activation does not indicate which protein(s) are actually unfolded/misfolded in the ER. Second, several studies have shown that the UPR branches can be differentially activated without a canonical "ER stress". Therefore, future studies on ER stress in IBD should directly measure unfolded/misfolded protein accumulation in IECs, especially Paneth and goblet cells. Protein misfolding and trafficking can be measured by analysis of post-translational modifications, including disulfide bond formation, glycosylation, and proteolytic cleavage. Previous studies have successfully employed immunoblotting and immunostaining approaches to study defective protein folding, maturation, and trafficking in goblet and Paneth cells. McGuckin et al. used antibodies against mature MUC2 mice as well as unglycosylated MUC2 precursor to examine folding, glycosylation, and secretion of MUC2 in goblet cell-like cell lines and primary colonic goblet cells in mice [36, 37]. Analysis of protein lysates by non-reducing electrophoresis on polyacrylamide gels can reveal aberrant disulfide bond formation in the ER. Ouellette et al. have used acetic acid-urea polyacrylamide gel electrophoresis (AU-PAGE) to examine the folding pattern of murine cryptdins, a family of antimicrobial peptides secreted by Paneth cells [38, 39]. Compared to the conventional biochemical techniques, novel sensitive glycomics chromatography/mass spectrometry technologies can provide more detailed information regarding alterations in specific glycan structures and their relative abundances. Recently, this technique was used to detect altered O-glycosylation profile of MUC2 mucin in

patients with active ulcerative colitis [40]. I believe the use of these techniques will further enhance our understanding of how ER stress affects IEC function and homeostasis.

Future directions for research on ER stress in IBD

Studies of ER stress in IBD are very new as indicated by only ~ 30 publications (including ~ 10 reviews) are presently in Pubmed. So far, only several UPR components have been studies in the intestinal epithelial compartment under normal conditions or using experimental colitis or enterobacterial infection. However, there is limited information regarding how the UPR pathways function in IECs upon challenge with different intestinal stresses, e.g. DSS-induced chronic colitis (Th2 dominant) or colitis in *Il10*$^{-/-}$ mice (Th1 dominant). In addition, lack of a good cell culture model (there are several "goblet cell-like" colon cancer cells available, but no Paneth cell lines are presently available, to the best of my knowledge) also impedes our understanding of the role of ER stress/UPR in IEC function and homeostasis upon inflammation. Given the difficulty of culturing isolated IECs *in vitro*, differentiation of primary intestinal stem cells into epithelial organoids that include Paneth and goblet cells may be a promising new direction in the future.

One cannot study IBD without studying inflammatory responses. Another exciting direction for the ER stress-IBD field is the role of ER stress/UPR in immune cells such as macrophages, neutrophils, NK cells, dendritic cells, T cells, and B cells during the pathogenesis of IBD, which is an almost untouched area. As mentioned earlier, the IRE1-XBP1 pathway regulates the differentiation and activation of several immune cells [41-57]. CHOP was recently shown to induce IL23 expression in dendritic cells, which

is critical for the Th17 response in the gut and pathogenesis of IBD [58]. Additionally, CHOP is important for caspase-11 activation and IL1β secretion in lung and cultured macrophages [59]. GADD34 may be important for differentiation and/or apoptosis of lymphocytes in mice (unpublished observations by the Kaufman group). In addition, it is unknown how the three IBD-associated genes *XBP1*, *AGR2*, and *ORMDL3* affect immune cell function in the inflamed gut. So far only one study directly examined the role of ER stress in T cell signaling in IBD, which suggested that the cytotoxicity of CD8αβ+ intraepithelial lymphocytes in the ileum of $Tnf^{\Delta ARE}$ mice was upregulated by genetically induced ER stress [60]. However, the authors used whole-body deletion of one *Bip* allele to induce ER stress in T cells, which raises the possibility that ER stress in the epithelial compartment, as well as other inflammatory cells, may have also contributed to the disease phenotype. Moreover, this study provides little information about how specific UPR pathways regulate the differentiation and activation of T cells during the progression of ileitis in mice. Future studies should employ conditional deletion/overexpression of specific UPR components in murine models of ileocolitis to investigate their impact on immune cell populations in the gut and other peripheral organs.

The last ten years have witnessed an increased understanding of how ER stress signals the UPR. With this armamentarium of knowledge, it is now possible to rationally design therapeutics to target protein-folding pathways and UPR signaling to resolve protein misfolding in disease states in a manner that was not previously conceivable. At least five types of strategies have been tested in cell culture, animal models, and clinical studies for various diseases (Figure 5-1). The first includes chemical chaperones that

prevent protein misfolding by stabilizing folding intermediates and preventing aggregation in the cell. The function of these compounds is considered non-specific to the folding substrate. A second type of small molecules enhance ER protein folding capacity and inhibit pro-apoptotic ER stress signaling by targeting specific UPR components, including ER stress sensors and transcription factors. The inhibitors of IRE1 and GADD34 belong to this group. The third type enforces the function of the proteostasis network by improving the microenvironment for ER protein folding, e.g. orchestrating ER Ca^{2+} flux and signaling. The last type of compounds alleviates ER stress and boosts protein-folding capacity by targeting related intracellular signaling pathways, including oxidative stress and autophagy. Despite promising preclinical data that support the notion that treatment with ER stress modulators mitigates IBD in animal models, there remains a large gap between bench studies and bedside use. Given the critical roles of some UPR pathways, particularly the IRE1-XBP1 and PERK-eIF2α pathways, in many organs/tissues, optimized dosing and timing are vital to achieve desired therapeutic effects, while avoiding adverse events. Intestine-specific drug delivery is another strategy to reach this end [61].

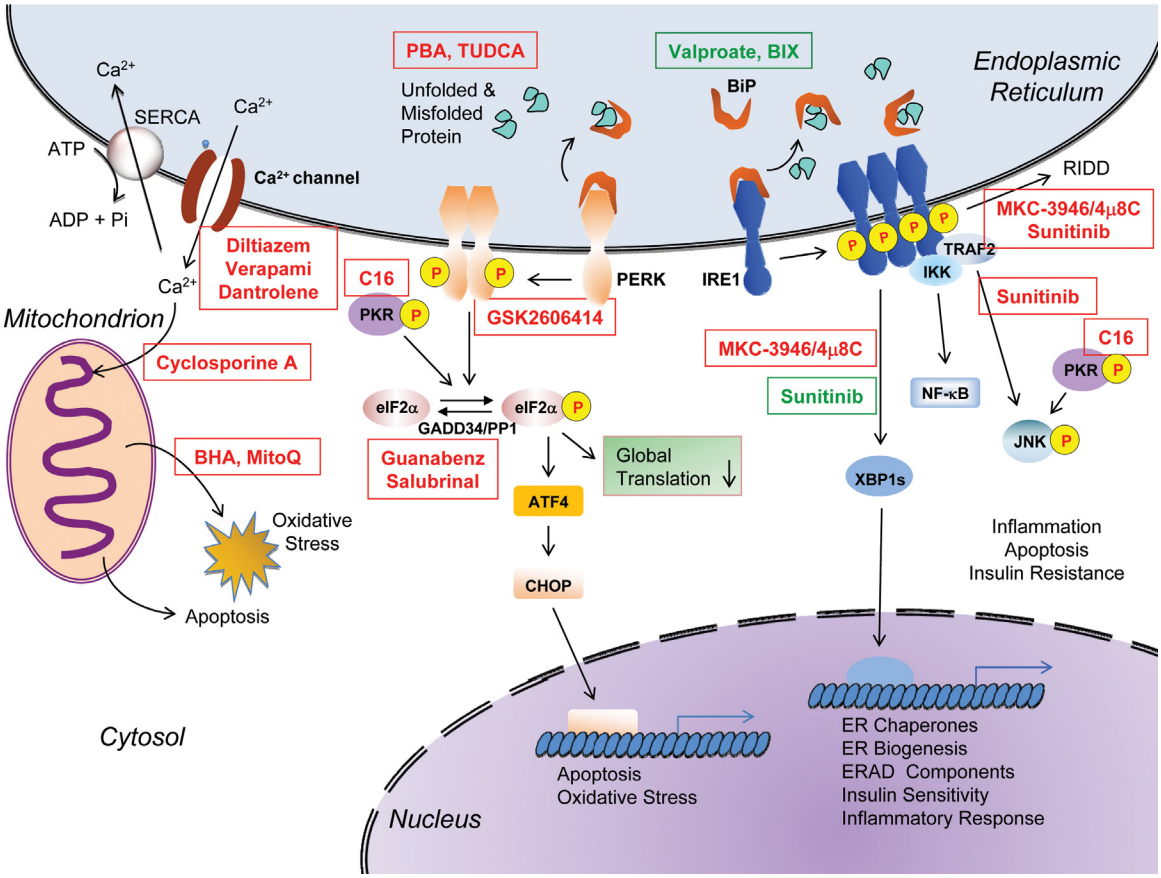

Figure 5-1: Targeting ER protein folding and the UPR with low molecular weight compounds. To date, a number of low molecular weight compounds have been developed/identified that target ER stress-related cellular processes and signaling pathways (Green - activators; Red - inhibitors). Among these compounds, chemical chaperones PBA and TUDCA that reduce ER protein misfolding in a non-specific manner, chemical inhibitors such as MKC-3946 and guanabenz that target specific UPR components, while cyclosporine A, butylated hydroxyanisole (BHA) and MitoQ that suppress mitochondrial dysfunction and oxidative stress or influence calcium signaling that are closely related to ER protein-folding homeostasis.

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