The orphan nuclear receptor SHP regulates ER stress response by inhibiting XBP1s degradation

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The orphan nuclear receptor SHP (small heterodimer partner) is a well-known transcriptional corepressor of bile acid and lipid metabolism in the liver; however, its function in other tissues is poorly understood. Here, we report an unexpected role for SHP in the exocrine pancreas as a modulator of the endoplasmic reticulum (ER) stress response. SHP expression is induced in acinar cells in response to ER stress and regulates the protein stability of the spliced form of X-box-binding protein 1 (XBP1s), a key mediator of ER stress response. Loss of SHP reduces XBP1s protein level and transcriptional activity, which in turn attenuates the ER stress response during the fasting–feeding cycle. Consequently, SHP-deficient mice also are more susceptible to cerulein-induced pancreatitis. Mechanistically, we show that SHP physically interacts with the transactivation domain of XBP1s, thereby inhibiting the polyubiquitination and degradation of XBP1s by the Cullin3–SPOP (speckle-type POZ protein) E3 ligase complex. Together, our data implicate SHP in governing ER homeostasis and identify a novel posttranslational regulatory mechanism for the key ER stress response effector XBP1.

[Keywords: SHP; exocrine pancreas; XBP1s; ER stress; Cullin3; ubiquitination]

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Small heterodimer partner [SHP; NR0B2] is an atypical orphan member of the nuclear receptor family that is best known for its role in hepatic bile acid and lipid metabolism [Lee et al. 2007; Chanda et al. 2008]. SHP lacks a canonical DNA-binding domain [DBD] and functions as a transcriptional repressor by interacting directly with other nuclear receptors and transcription factors [Båvner et al. 2005]. In hepatocytes, Shp expression is induced by the bile acid-activated nuclear receptor FXR [NR1H4], and, in turn, SHP interacts with nuclear receptors liver receptor homolog-1 [LRH-1; NR5H2] and hepatocyte nuclear factor factor 4a (HNF4a; NR2A1) to repress the expression of enzymes mediating bile acid synthesis [Goodwin et al. 2000, Lu et al. 2000, Kir et al. 2012]. In addition, SHP regulates macrophage inflammation, enteric cholesterol absorption, and gonadal steroidogenesis and suppresses tumorigenesis [Volle et al. 2007; Zhang et al. 2008; Yuk et al. 2011; Kim et al. 2019]. Interestingly, while Shp mRNA has been detected in the pancreas [Sanyal et al. 2002], its function there remains unclear.

A key step in nutrient digestion is the synthesis and secretion of digestive enzymes by acinar cells in the exocrine pancreas. The endoplasmic reticulum (ER) is the site for protein synthesis and folding of these digestive enzymes [Logsdon and Ji 2013]. During the cycle of fasting and feeding, acinar cells produce more protein than any other cell type in the body. Acinar cells adapt to this dynamic demand in protein synthesis through a universal and highly conserved quality control mechanism known as the unfolded protein response (UPR) to reset ER folding capacity and homeostasis [Walter and Ron 2011]. In mammals, the UPR is initiated through the activation of three ER-resident sensors: inositol-requiring enzyme 1α [IRE1α], activating transcription factor 6 [ATF6], and protein kinase R [PKR]-like ER kinase [PERK] [Logsdon and Ji 2013].

IRE1α initiates the most conserved UPR signaling pathway. Upon sensing the accumulation of misfolded...
proteins in the ER, activated IRE1α splices 26 nucleotides (nt) from the unspliced X-box-binding protein 1 (Xbp1u) mRNA, leading to the generation of an active transcription factor, XBP1s [spliced XBP1] (Logsdon and Ji 2013). XBP1s contains a C-terminal transactivation domain (TAD), allowing it to translocate to the nucleus and induce transcription of genes involved in protein folding and degradation in the ER (Logsdon and Ji 2013). Studies in animal models have revealed the essential role of XBP1s in the exocrine pancreas. The IRE1α–XBP1s pathway is constitutively activated in the exocrine pancreas and is further activated during the fasting–feeding cycle (Yang et al. 2010). Mice deficient in Xbp1 exhibit severe exocrine pancreatic insufficiency, leading to early postnatal lethality (Lee et al. 2005). Xbp1 heterozygous mice are prone to alcohol-induced pancreatic damage [Lugea et al. 2011]. Together, these results establish the crucial role of the IRE1α–XBP1s pathway in maintaining ER homeostasis and physiological functions of the exocrine pancreas.

XBP1s transcriptional activity can be regulated further by posttranslational modifications such as phosphorylation, acetylation, and sumoylation (Chen and Qi 2010; Wang et al. 2011; Liu et al. 2016). Moreover, a recent study showed that XBP1s protein stability can also be modulated in natural killer cells in response to interleukin-15 signaling (Wang et al. 2019). How XBP1s protein is degraded in other cell types or tissues remains unclear. Here, we report a positive feedback regulatory mechanism between SHP and XBP1s, wherein Shp expression is induced by XBP1s in response to ER stress. SHP in turn stabilizes XBP1s protein and thereby enhances its activity by inhibiting the Cullin3–SPOP [speckle-type POZ protein] E3 ligase complex that degrades XBP1s. Our findings reveal an unexpected role for SHP as an important regulator of the ER stress response in the exocrine pancreas and identify an E3 ligase complex responsible for XBP1s protein turnover.

Results

SHP expression in the pancreas

Determining in which tissues SHP is expressed has been severely hampered by the lack of SHP-specific antibodies. To circumvent this issue, we generated a knock-in mouse model using CRISPR/Cas9 technology that expresses three Flag tags in tandem at the N terminus of SHP (Fig. 1A). In addition to the liver, SHP protein was highly expressed in the pancreas (Fig. 1A). As a previous two-hybrid screen identified a physical interaction between SHP and XBP1 (Ravasi et al. 2010) and since XBP1s is a key regulator of ER homeostasis in the pancreas [Lee et al. 2005], we hypothesized that SHP may be involved in the regulation of ER homeostasis via XBP1s in the pancreas.

XBP1s induces Shp gene transcription in pancreatic acinar cells

ER stress-inducing agents such as thapsigargin (Tg) and dithiothreitol (DTT) strongly stimulated Shp expression in the pancreas in vivo and in AR42J acinar cells and primary acinar cells in vitro (Fig. 1B–D). We next determined how ER stress induces Shp. The expression of Shp in acinar cells during ER stress was attenuated by treatment with the IRE1α-specific inhibitor 4µ8C (Fig. 1E; Supplemental Fig. S1A; Cross et al. 2012). As expected, 4µ8C treatment also down-regulated the expression of the XBP1s target genes Erdj4, Hrd1, and Bip but not the PERK downstream target Chop (Fig. 1F; Supplemental Fig. S1B). Deletion of Xbp1 in AR42J cells [Supplemental Fig. S1C] abolished the induction of Shp expression by ER stress (Fig. 1G). Xbp1 deletion also reduced the expression of other IRE1α–XBP1s target genes such as Erdj4, Hrd1, and Bip, but not Chop, in response to ER stress (Fig. 1H; Supplemental Fig. S1D). Taken together, these data suggest that the IRE1α–XBP1s signaling pathway directly regulates Shp expression in response to ER stress.

We next determined whether XBP1s directly regulates Shp gene transcription. Computational analysis of the mouse Shp gene promoter revealed a putative XBP1s-binding site [Acosta-Alvear et al. 2007] at nucleotide −193 upstream of the transcription initiation site that is conserved in rats and humans [Fig. 1I]. To establish that XBP1s acts through this site, we constructed mouse Shp promoter (−492 to +36 bp) luciferase reporters with wild-type [WT], mutant, or deleted XBP1s-binding sites (Fig. 1J) and performed cotransfection assays in HEK293T cells expressing XBP1s. Overexpression of XBP1s enhanced luciferase expression driven by the WT Shp promoter, which was significantly attenuated when the XBP1s-binding site was mutated or deleted [Fig. 1J,K]. Chromatin immunoprecipitation [ChIP] experiments showed enrichment of XBP1s binding around this element (−190 bp) but not at control sites either upstream of (−2600 bp) or downstream from (−2400 bp) the Shp promoter. As a positive control, we detected XBP1s binding to the Bip promoter at −100 bp but not −2800 bp as reported previously [Yoshida et al. 1998]. Collectively, these data demonstrate that Shp is transcriptionally induced by ER stress in pancreatic acinar cells via the IRE1α–XBP1s signaling pathway.

SHP regulates the ER stress response during fasting/refeeding

We next investigated whether SHP plays a physiologic role in UPR signaling in the exocrine pancreas. To that end, we examined the induction of pancreatic UPR in Shp knockout mice during fasting and refeeding. In line with previous studies [Yang et al. 2010], UPR genes were induced in the pancreas of WT mice after refeeding for 2 h following an overnight fast [Fig. 2A,B]. Notably, the induction of XBP1s target genes, including Hrd1, Bip, Pdia6, and Erdj4, was either significantly reduced or abolished in the pancreata of Shp knockout mice [Fig. 2A]. Shp deficiency had no effect on the PERK signaling pathway, as evidenced by the lack of effect of Shp deficiency on Chop gene expression [Fig. 2B].

To dissect how Shp deficiency down-regulates the IRE1α–XBP1 pathway during ER stress, we analyzed the splicing of Xbp1 mRNA and its protein level. While
Xbp1 mRNA splicing by IRE1α in response to feeding was comparable between WT and Shp knockout mice (Fig. 2C), the induction of XBP1s protein levels after refeeding was abolished in the Shp knockout pancreas (Fig. 2D). Shp heterozygous mice were indistinguishable from their WT littermates, suggesting that one copy of Shp is sufficient to maintain XBP1s protein level and activity (Fig. 2A–C). Together, these data indicate that SHP modulates ER homeostasis in the pancreas by posttranslationally regulating XBP1s protein levels.

Because SHP also is highly expressed in the liver (Fig. 1A), we examined whether SHP similarly regulates hepatic XBP1s signaling. Intraportal injection of tunicamycin (Tun) strongly induced the ER stress response in the liver (Supplemental Fig. S2A,B). Similar to the pancreas, the induction of XBP1s target genes, but not Chop, was attenuated in Shp knockout livers during ER stress (Supplemental Fig. S2A,B). While IRE1α-mediated Xbp1 mRNA splicing was comparable between WT and Shp knockout mice, the induction of XBP1s protein was markedly reduced in the Shp knockout liver in response to ER stress (Supplemental Fig. S2C,D). These results suggest a broader role for SHP in regulating XBP1s protein level and activity.

Shp-deficient mice are prone to cerulein-induced pancreatitis (CIP)

The IRE1α–XBP1s pathway is important for not only maintaining the normal digestive function of the pancreas but also protecting against pancreatitis (Lee et al. 2005; Lugea et al. 2011). To test whether SHP plays a role in this protection, pancreatitis was induced in mice by six hourly injections of cerulein followed by measurement of pancreatic markers. Relative to WT mice, Shp knockout mice were sensitized to CIP, as evidenced by elevated plasma levels of amylase (Fig. 3A), increased transcription of inflammatory markers and Chop (Fig. 3B), and aggravated edema and cell death in the pancreas (Fig. 3C). Note that the elevated expression of Chop is likely secondary to elevated tissue inflammation and cell death during CIP; thus, it was not observed in the previous experiments.
These data show that SHP plays an important role in protecting the pancreas from cellular stress and inflammation during pancreatitis.

To investigate how SHP regulates XBP1s protein and activity, we first examined whether the two proteins physically interact. In transfected HEK293T cells, XBP1s was readily immunoprecipitated by HA-tagged SHP (Fig. 4A). Using the 3XFlag-Shp knock-in mice, we also observed that SHP immunoprecipitated XBP1s in the pancreas under refed conditions (Fig. 4B). To map the domain in XBP1s that mediates its interaction with SHP, we generated XBP1s truncations by deleting either the N-terminal DBD or portions of the C-terminal TAD (Fig. 4C; Yoshida et al. 2001; Lee et al. 2002). Interestingly, the last 70 amino acids (301–371) of the C-terminal TAD of XBP1s were required for its interaction with SHP (Fig. 4D).

Given that XBP1s is a highly unstable protein (Calfon et al. 2002), we speculated that SHP might regulate XBP1s protein levels by modulating its degradation. Indeed, overexpression of XBP1s polyubiquitination in transfected HEK293T cells (Fig. 5A). To directly measure the XBP1s protein turnover rate, we induced endogenous XBP1s by Tuni injection with or without the protein translation inhibitor cycloheximide (CHX). XBP1s was degraded faster in Shp knockout than in WT mice (Fig. 5B). Thus, SHP interacts with and stabilizes XBP1s protein by attenuating its ubiquitination.

The Cullin3–SPOP E3 ligase complex mediates XBP1s ubiquitination

We next explored how SHP regulates XBP1s ubiquitination. Treatment with the proteasomal inhibitor MG132 stabilized XBP1s and led to its accumulation, as expected (Fig. 6A, lanes 1–3). Intriguingly, treating cells with MLN4924, an inhibitor targeting the Cullin-based family of E3 ligases (Brownell et al. 2010; Liao et al. 2011), also stabilized XBP1s protein (Fig. 6A, lanes 4–6), although not to the same extent as MG132. These results suggest that Cullins may be one class of E3 ligases that can mediate XBP1s degradation.

The Cullin-based E3 ligases comprise the largest known family of ubiquitin ligases. Cullin proteins function as scaffolds for the assembly of the multiunit E3 ligase complexes, within which specific adaptor proteins confer substrate specificity (Petroski and Deshaies 2005). In order to identify Cullin family members involved in XBP1s ubiquitination, we examined which Cullins interact physically with XBP1s. Among the seven Cullin members, Cullin1, Cullin3, and Cullin4B strongly interacted with XBP1s in transfected HEK293T cells (Fig. 6B). Further analysis revealed that Cullin3 alone induced some polyubiquitination of XBP1s (Fig. 6C). Notably, however, the addition of SPOP, an adaptor protein that recruits protein substrates to Cullin3 (Zhuang et al. 2009), markedly enhanced Cullin3-mediated XBP1s ubiquitination (Fig. 6D). Collectively, these results establish that the Cullin3–SPOP E3 ligase complex can mediate XBP1s ubiquitination.

To establish that Cullin3 mediates XBP1s degradation, we measured endogenous XBP1s protein decay in cells with or without Cullin3. Deletion of Cullin3, but not
Cullin1 or Cullin4B, in both HEK293T cells and 266-6 pancreatic acinar cells attenuated XBP1s protein degradation (Fig. 6E; Supplemental Fig. S3A,B). In addition, XBP1s physically interacted with both endogenous Cullin3 and SPOP proteins in both cell types (Fig. 6F; Supplemental Fig. S3C). Thus, the Cullin3–SPOP complex mediates XBP1s protein ubiquitination and degradation.

SHP attenuates Cullin3-mediated XBP1s ubiquitination

Given that SHP inhibits and Cullin3–SPOP induces XBP1s ubiquitination, we next asked whether SHP inhibits the activity of Cullin3/SPOP. First, we analyzed the subcellular localization of these proteins using fractionation and immunocytochemistry. XBP1s, SHP, Cullin3, and SPOP all localized in the nucleus [Fig. 7A; Supplemental Fig. S4]. By immunostaining, SHP, Cullin3, and SPOP all colocalized with XBP1s to varying degrees [Fig. 7B; Supplemental Fig. S4]. Next, we investigated whether SHP inhibits Cullin3–SPOP-mediated XBP1s ubiquitination. Overexpression of SHP strongly attenuated Cullin3–SPOP-induced XBP1s polyubiquitination (Fig. 7C). Interestingly, similar to SHP, the Cullin3–SPOP complex physically interacted with amino acid residues 301–371 of the C-terminal TAD of XBP1s (Fig. 7D), suggesting that SHP and Cullin3–SPOP compete for XBP1s binding. Indeed, overexpression of SHP effectively inhibited the interaction between XBP1s and Cullin3–SPOP (Fig. 7E). In contrast, Cullin3–SPOP had no effect on the interaction between XBP1s and SHP (Fig. 7E). These data suggest that SHP directly blocks the Cullin3–SPOP E3 ligase complex from interacting with XBP1s, thereby stabilizing XBP1s.

Discussion

In this study, we identified a feedback regulatory loop between SHP and XBP1s that governs ER stress in the pancreas (Fig. 7F). We demonstrate that Shp is a direct transcriptional target of XBP1s in the exocrine pancreas, which in turn regulates the protein stability of XBP1s by inhibiting its polyubiquitination. We further show that this positive regulatory loop is an important part of the normal physiological response to the cycle of fasting and refeeding and that it protects against the pathogenesis of pancreatitis.

XBP1s plays a central role in maintaining ER homeostasis and physiological functions of the exocrine pancreas.

Cullin1 or Cullin4B, in both HEK293T cells and 266-6 pancreatic acinar cells attenuated XBP1s protein degradation [Fig. 6E; Supplemental Fig. S3A,B]. In addition, XBP1s physically interacted with both endogenous Cullin3 and SPOP proteins in both cell types [Fig. 6F; Supplemental Fig. S3C]. Thus, the Cullin3–SPOP complex mediates XBP1s protein ubiquitination and degradation.

SHP regulates Cullin3-mediated XBP1s degradation

Figure 4. SHP interacts with the C-terminal TAD of XBP1s. (A) Western blot analysis of immunoprecipitates of Flag-agarose in HEK293T cells transfected with the indicated plasmids, showing the interaction between XBP1s and SHP. (B) Western blot analysis of immunoprecipitates of Flag-agarose in pancreatic lysates from WT and 3XFlag-SHP knock-in (Flag-ShpKI) mice after an overnight fast and/or 4 h of refeeding, showing the interaction between endogenous XBP1s and SHP under a physiological setting. (C) Truncated mutants of XBP1s used in D. (D) Western blot analysis of immunoprecipitates of Flag-agarose in HEK293T cells transfected with the indicated plasmids, showing the interaction between variants of XBP1s and SHP. Numbers indicate amino acid positions.
Xbp1 heterozygous mice, which have half the amount of XBP1s protein in the pancreas, develop more severe alcohol-induced pancreatitis associated with elevated expression of the proapoptotic gene Chop [Lugea et al. 2011]. In this study, we demonstrated that Shp knockout mice were also prone to CIP associated with elevated Chop expression (Fig. 3). The phenotype of Shp knockout mice is consistent with the known prosurvival function of XBP1s (Romero-Ramirez et al. 2004; Gupta et al. 2010) and with the phenotype of Xbp1 heterozygous mice. Interestingly, under basal conditions, Shp knockout mice appear normal in terms of XBP1s protein level (Fig. 2), pancreatic function, and inflammation (Fig. 3), pointing to additional transcriptional or translational mechanisms regulating XBP1s level. Nevertheless, our findings demonstrate that a major role of SHP in the exocrine pancreas is to govern stress-induced XBP1s function. A previous report using single-cell sequencing analysis suggested that SHP is expressed in both the exocrine pancreas and the endocrine pancreas [Segerstolpe et al. 2016]. Thus, it is likely that SHP may have additional XBP1s-independent actions in the pancreas.

The XBP1s protein is known to be unstable [Calfo et al. 2002]; however, the underlying mechanism remains poorly understood. A recent study suggested that PGC1α represses XBP1s protein level in the liver by promoting its ubiquitination and degradation [Lee et al. 2018]. Interestingly, this study mapped amino acid residues 227–252 of XBP1s as required for its interaction with PGC1α [Lee et al. 2018], distinct from residues 301–371, which are required to interact with SHP. Very recently, another study showed that XBP1s ubiquitination and protein stability in natural killer cells are modulated in response to interleukin-15 signaling [Wang et al. 2019]. An outstanding question has been the nature of the E3 ligase that mediates XBP1s degradation. Here, we show that the Cullin3–SPOP complex is a strong candidate for one of the E3 ligase activities. Future studies are required to determine whether the Cullin3/SPOP-mediated degradation of XBP1s is regulated by physiological and pathological signals such as fasting, feeding, and inflammation.

We showed previously that Shp is transcriptionally induced by the FXR/RXR nuclear receptor heterodimer in response to bile acid stimulation in the liver, where it functions as a repressor in the feedback regulatory loop that governs bile acid synthesis [Goodwin et al. 2000; Lu et al. 2000]. In this study, we report the presence of a conserved XBP1s response element that is distinct from the bile acid/FXR response element in the SHP promoter, suggesting a distinct mode of regulation in response to ER stress. Thus, it is intriguing to compare the differences between bile acid and XBP1s signaling in the exocrine pancreas. Bile acid reflux into the pancreatic duct is proposed to be a triggering event of biliary pancreatitis [Lerch and Aghdassi 2010]. It has been shown that bile acids induce acinar cell injury by eliciting calcium release from the ER into the cytosol, which also triggers ER stress [Kim et al. 2002; Voronina et al. 2002; Mekahl et al. 2011]. Notably, however, we failed to induce Shp transcription or UPR in vitro by treating AR42J acinar cells with bile acids or the FXR agonist GW4064 [data not shown], further suggesting that the two pathways are distinct. In the future, it will be worth investigating whether bile acids can coordinate with UPR signaling to regulate Shp expression and modulate ER homeostasis in normal pancreatic physiology and during pancreatitis.

In summary, our study establishes the importance of the orphan nuclear receptor SHP in modulating ER homeostasis in vivo. Shp is transcriptionally induced by XBP1s in response to ER stress and in turn regulates the stability of XBP1s by inhibiting the activity of the Cullin3–SPOP E3 ligase complex. We further established the physiological importance of this regulatory mechanism for maintaining normal pancreatic physiology and protecting against the pathogenesis of pancreatitis. This work expands the tissue-specific actions of SHP and the regulation of XBP1 and highlights the cross-talk between these two important signaling pathways.
Materials and methods

Mice

Shp knockout mice [Kerr et al. 2002] and WT littermates were maintained on a C57BL/6J background. For fasting–refeeding experiments, mice were fasted overnight and refed for 2 h with standard chow diet. To induce XBP1s and measure its decay in vivo, mice were injected intraperitoneally with DTT (Sigma, D9779) at 0.75 mmol per kilogram of body weight for 6 h, Tuni (Tocris, 3516) at 1 mg per kilogram of body weight for 4 h, and/or CHX (Millipore, 508739) at 2 mg per kilogram of body weight for 20 min. Acute pancreatitis was induced by the administration of six hourly intraperitoneal injections of cerulein (Tocris, 6264) at 50 mg per kilogram of body weight each time, and tissues were collected 1 h after the last injection. Plasma amylase activity was measured using amylase assay kit per the manufacturer’s instructions (Abcam, ab102523). For histology, the pancreas was fixed in 10% neutral-buffered formalin overnight, paraffin-embedded, sectioned, and H&E-stained. Images were acquired with a Zeiss AxioScope Z1 slide scanner, and necrotic cell percentages were quantified by counting the number of necrotic cells and total cells from three random fields of the tissue section of each mouse.

3XFlag-Shp knock-in mice were generated with the CRISPR/Cas9 method [Yang et al. 2013]. A 66-nt sequence (5’-GACTA CAAAGACCACACGAGTGACTACAAGGACCACGACATC GACTACAAGGACGACGACAAG-3’) encoding a 3XFlag

Figure 6. Cullin3 is an XBP1s E3 ligase. (A) Western blot analysis of XBP1s in HEK293T cells transfected with an XBP1s-Flag-expressing plasmid and treated with 10 µM MG132 or 1 µM Cullin inhibitor MLN4924 for 0, 2, or 4 h. Quantification of XBP1s protein levels is shown at the right. Data are represented as mean ± SEM. (B) Western blot analysis of immunoprecipitates of Flag-agarose in HEK293T cells transfected with the indicated plasmids, showing the interaction between XBP1s and Cullin family members. (C) Western blot analysis of immunoprecipitates of Myc-agarose in HEK293T cells transfected with the indicated plasmids, showing XBP1s ubiquitination by Cullin3 alone (C, lane 3) or Cullin3 and SPOP (D, lane 3). In C, the level of XBP1s ubiquitination is quantified below the blot. (E) Western blot analysis of endogenous XBP1s decay in Cullin3 knockout 266-6 acinar cells [knockout [KO]] or control cells [CON] with a nonspecific targeting guide, treated with 2.5 nM Tg for 2.5 h followed by 150 µM CHX for 1 h. Quantification is shown at the right. Data are represented as mean ± SEM. (F) Western blot analysis of immunoprecipitates of Flag-agarose in 266-6 cells transfected with XBP1s-Flag-expressing plasmid showing the interaction between XBP1s and endogenous Cullin3 and SPOP. In B–D and F, cells were treated with proteasomal inhibitor MG132 for the last 3 h prior to immunoprecipitation.
tag was inserted in-frame into the 5′ end of the endogenous SHP-coding region right after the translation start codon. A single guide RNA with sequence 5′-TGGGCAGACCCCTGACTGGC-3′ was microinjected into C57BL/6J zygotes in conjunction with the Cas9 mRNA (Trilink Bio) and a single-stranded homology-directed repair (HDR) template containing the 66-nt 3XFlag-coding sequence flanked by the left (66-nt) and right (65-nt) arms of homologous sequence (IDT Ultramer). The injected zygotes were implanted into pseudopregnant female C57BL/6J mice. Mice born from these microinjections were screened by PCR from tail DNA to verify HDR template insertion, and germline transmission was confirmed in the second generation of breeding with WT C57BL/6J mice to eliminate any potential mosaicism. For this study, male homozygous Shp knock-in mice and their WT littermates were used.

Male mice were used for all experiments. All animal experiments were approved by the Institutional Animal Care and Research Advisory Committee of the University of Texas Southwestern Medical Center. Mice were housed in a pathogen-free and temperature-controlled environment with 12-h light/dark cycles (6 am–6 pm) and fed standard irradiated rodent chow (Harlan Teklad, 2916).

Isolation of primary acinar cells

Primary mouse acinar cells were prepared as described previously (Coate et al. 2017). Briefly, pancreata were dissected, rinsed, and cut in ice-cold Hank’s buffered salt solution (HBSS) into 1- to 2-mm pieces. The pancreas pieces were then digested with digestion medium (0.75 mg/mL collagenase type I-A [Sigma, C2674], 0.1 mg/mL soybean trypsin inhibitor [Sigma, T6522], 1% BSA, 10% FBS in DMEM) for 35 min at 37°C with constant mixing using serological pipets. Cells were then washed three times with washing buffer [10% FBS, 1% penicillin–streptomycin in DMEM], resuspended in incubation medium (0.1 mg/mL soybean trypsin inhibitor, 1% BSA, 10% FBS, 1% penicillin–streptomycin in DMEM), and filtered through a 100-μm strainer. Cells were plated in incubation medium and allowed to recover for 30 min before the 2 mM DTT treatment for 4 h.

Cell culture

Rat AR42J acinar cells were cultured in DMEM (Gibco) containing 20% heat-inactivated FBS [VWR] and 1% penicillin–streptomycin [Gibco]. For RNA or protein analysis, cells were treated
Lenti-X 293T cells (Clontech) were transfected with plasmids Millipore. 412512 for 4 h. For CRISPR knockouts of Xbp1, Lenti-X 293T cells [Clontech] were transfected with plasmids LentiCRISPR-v2 [Addgene, 52961], pPAx2 [Addgene, 12260], and pMD2 [Addgene, 12259] using FuGene HD reagent (Promega, E2311) to generate lentivirus carrying a nontargeting control guide [5′-GATCGTTTCGCCGTTAACGCGC-3′] or guides targeting rat Xbp1 [5′-CCGGCGCGGGAGCAAATGAGCG-3′ and 5′-TTCCGGGCCCCGAGGCCGCA-3′]. AR42J cells were transduced with lentivirus in the presence of 8 μg/mL polybrene [Millipore, TR-1003-G], selected with 2 μg/mL puromycin for 2 d, and analyzed 8 d after transduction.

HEK293T cells were cultured in DMEM (Gibco) containing 10% FBS and 1% penicillin-streptomycin and transfected with various plasmids using 80 μg/μL polyethylenimine [PEI, Sigma, 408727]. After transfection, HEK293T cells were treated with 10% FBS and 1% penicillin-streptomycin using 80 μg/μL polyethylenimine [PEI, Sigma, 408727]. For immunoprecipitation, cells or tissue were lysed by sonication in a buffer containing 150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1% NP-40, protease inhibitors, and 5 mM N-ethylmaleimide. A total of 2–5 μg of protein lysates was precleared with protein G agarose for 2 h at 4°C and then incubated with anti-Flag M2 agarose [Sigma, A2220] or anti-Myc agarose [Cell Signaling, 3400] overnight at 4°C with gentle rocking. Immunocomplexes were washed four times in the lysis buffer and eluted by boiling for 5 min at 95°C in SDS sample buffer.

**Western blot**

Western blot was performed using 10–30 μg of total protein lysates or 20 μg of nuclear extracts. The following antibodies were used in this study: XBP1s [Cell Signaling, 83418], CHOP [Cell Signaling, 2895], PARP [Cell Signaling, 9532], ubiquitin [Cell Signaling, 3933], Cullin1 [Cell Signaling, 4995], Cullin3 [Cell Signaling, 2759], Cullin4B [ProteinTech, 12916-1-AP], SPOP [ProteinTech, 16750-1-AP], Flag [Sigma, F1804 and F7425], Myc [Cell Signaling, 2278 and 2276], HA [Sigma, H3663, and Cell Signaling, 3724], β-Actin-HRP [Abcam, ab49000], and HSP90 [Santa Cruz Biotechnology, sc7947]. Secondary antibodies—donkey antirabbit or mouse IgG HRP [Jackson ImmunoResearch]—were used at 1:6000 dilution. Western blot membranes were developed using the Clarity Western ECL substrate [Bio-Rad], and signal was detected with an ImageQuant LAS4000 luminescent imager [General Electric]. Quantification was performed using ImageQuant TL software [General Electric].

**Nuclear fractionation**

Nuclear fractionation was performed as described previously [Sha et al. 2009]. Briefly, liver or pancreatic tissues were lysed in a hypotonic buffer [10 mM HEPES at pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, protease inhibitors] with dounce tissue grinders [DKW Life Sciences, 885303-0002] and allowed to swell for 15 min on ice followed by the addition of 10% NP-40 to a final concentration of 0.6%. After being vortexed for 15 sec, the lysate was centrifuged at 12,000g for 5 min at 4°C. Supernatant was collected as the cytosolic fraction. Nuclear pellets were resuspend in high-salt buffer [20 mM HEPES at pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT] and vortexed vigorously for 15 sec every 5 min for a total of 20 min. Extracts were centrifuged, and the supernatant was collected as the nuclear fraction.

**RNA extraction, RT-PCR for Xbp1 mRNA splicing, and quantitative real-time PCR**

Pancreatic RNA was isolated as described previously [Sun et al. 2014; Coate et al. 2017]. Briefly, mice were anesthetized, and pancreatic tissues were perfused locally with RNAlater reagent [Qia-gen, 76104]. Pancreatic tissues were then excised and soaked in RNAlater on ice for stabilization. Liver tissues were snap-frozen in liquid nitrogen for RNA extraction. Total RNA from tissues and cultured cells was extracted using RNA-Stat 60 [Isotex Diagnostics] and RNA purification kit [Omega Biotek, R683402] with DNase I digestion [Roche]. RNA quality was determined by measuring the OD260/280 and visualization on an agarose gel. cDNA was generated using the high-capacity cDNA reverse transcription kit [Life Technologies]. RT-PCR for Xbp1 mRNA splicing was performed as described previously [Sha et al. 2009] using GoTag Green master mix [Promega] and an annealing temperature of 58°C for 35 cycles. PCR products were separated by electrophoresis on a 2% agarose gel. The percentage of Xbp1 mRNA splicing, defined as the ratio of Xbp1 splicing level to total Xbp1 [Xbp1u + Xbp1s] level, was quantified using ImageQuant TL software.
ChIP

266-6 acinar cells were transfected with pcDNA3-Flag-mXBP1s or empty plasmid using Lipofectamine 2000 reagent (Thermo Fisher, 11668019). Twenty-four hours after transfection, cells were cross-linked by 1% formaldehyde for 10 min at room temperature. ChIP was performed using SimpleChIP plus enzymatic ChIP kit with magnetic beads (Cell Signaling, 9005) following the protocol provided. Immunoprecipitation was performed with normal rabbit IgG (Cell Signaling, 2729) or ChIP-grade anti-Flag antibody (rabbit; 1:200, Cell Signaling, 2278) or anti-HA (rabbit; 1:200, Cell Signaling, 3724) primary antibodies followed by donkey anti-mouse-488 and donkey antirabbit-594 secondary antibodies (donkey antimouse-488 and donkey antirabbit-594 secondary antibodies (ThermoFisher, 1:200)). Immunoprecipitates were analyzed by qPCR using primers designed to amplifying 100 bp sequences around the TATA box.

Prolong Gold anti fade reagent with DAPI (Invitrogen). Fluorescent images were taken under a Zeiss LSM700 confocal microscope. The level of colocalization was quantified by the Pearson’s correlation coefficient (r) using ImageJ software with Costes autothreshold determination method for colocalization analysis.

Statistical analyses

Unpaired two-tailed Student’s t-test was used for two-group analyses. Two-way ANOVA analysis was used for multigroup analyses (GraphPad Prism). Data are presented as the mean ± SEM; P < 0.05 was considered significant.

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