Fic-mediated AMPylation tempers the unfolded protein response during physiological stress

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The proper balance of synthesis, folding, modification, and degradation of proteins, also known as protein homeostasis, is vital to cellular health and function. The unfolded protein response (UPR) is activated when the mechanisms maintaining protein homeostasis in the endoplasmic reticulum become overwhelmed. However, prolonged or strong UPR responses can result in elevated inflammation and cellular damage. Previously, we discovered that the enzyme filamentation induced by cyclic-AMP (Fic) can modulate the UPR response via posttranslational modification of binding immunoglobulin protein (BiP) by AMPylation during homeostasis and deAMPylation during stress. Loss of fic in Drosophila leads to vision defects and altered UPR activation in the fly eye. To investigate the importance of Fic-mediated AMPylation in a mammalian system, we generated a conditional null allele of Fic in mice and characterized the effect of Fic loss on the exocrine pancreas. Compared to controls, Fic−/− mice exhibit elevated serum markers for pancreatic dysfunction and display enhanced UPR signaling in the exocrine pancreas in response to physiological and pharmacological stress. In addition, both fic−/− flies and Fic−/− mice show reduced capacity to recover from damage by stress that triggers the UPR. These findings show that Fic-mediated AMPylation acts as a molecular rheostat that is required to temper the UPR response in the mammalian pancreas during physiological stress. Based on these findings, we propose that repeated physiological stress in differentiated tissues requires this rheostat for tissue resilience and continued function over the lifetime of an animal.

AMPylation | Fic | unfolded protein response | pancreas | ER stress

Protein homeostasis is regulated by proper synthesis, folding, modification, and degradation of proteins and is vital to cellular health. In the endoplasmic reticulum (ER), when the load of unfolded proteins is excessive, the unfolded protein response (UPR) is activated, triggering signaling pathways that result in changes to protein synthesis, modification, and degradation until the load of unfolded proteins is resolved. If the burden of unfolded proteins is prolonged and/or remains high, proapoptotic pathways can be activated. The activation of the UPR is, in part, regulated by the Hsp70 protein chaperone binding immunoglobulin protein (BiP), a protein that binds and helps fold proteins as they pass through the ER checkpoint and into the secretory pathway. Depending on the level of unfolded protein, complex signaling networks are activated and respond in accordance to the severity. Mild to moderate levels of UPR signaling are prominent with cell recovery and cell survival, whereas strong and prolonged UPR signaling leads to apoptosis (1,2). These responses are mediated by three distinct ER signaling branches: inositol-requiring enzyme-1α (IRE1α); protein kinase R-like ER kinase; and activating transcription factor 6 (Atf6).

In addition, the UPR stress can be divided into two phases: the adaptive phase and the maladaptive phase (4,5). For the adaptive phase, the UPR induction responds to mild to moderate stress and promotes prosurvival and restorative mechanisms to promote ER homeostasis (4). By contrast, the maladaptive phase is induced by chronic and severe ER stress resulting in the activation of proinflammatory responses and apoptosis (4,6). Disruption of ER homeostasis is predicted to play a key role in the integrated stress response and the progression of many neurodegenerative, inflammatory, and metabolic disorders (7-9). Elucidating the roles that the UPR plays in modulating ER stress provides potential therapeutic targets to treat or prevent the death of the cells subjected to prolonged ER stress and ameliorate UPR-related degenerative diseases.

Previously, the activity of BiP was shown to be regulated by a posttranslational modification (PTM) called AMPylation (10). AMPylation is a reversible PTM best described as the covalent linkage of adenosine monophosphate (AMP) to the hydroxyl group of a serine, threonine, or tyrosine residue (11). Initially discovered in the 1960s with a nucleotidyl transferase domain (12), protein AMPylation was rediscovered in 2009 with a

Significance

During an animal’s life span, terminally differentiated cells must be resilient to fluctuating environmental and physiological stresses to assure proper function of a tissue and prevent disease. A key component of this resiliency is the maintenance of protein homeostasis. Our study reveals that the AMPylating enzyme filamentation induced by cyclic-AMP (Fic) is required in differentiated tissue for the regulation of the unfolded protein response (UPR) during both physiological and pharmacological stresses. We also propose that cells with high regenerative capacity may not require this level of regulation, as new cells will bypass the need for long-term survival. We predict that Fic is important in mitigating deleterious effects of UPR activation in a variety of tissues with UPR-associated diseases and thus holds promise as a new therapeutic target.
filamentation induced by cyclic-AMP (Fic) domain from a bacterial pathogen that is also conserved in eukaryotic organisms (13, 14). To date, only two AMPylating enzymes have been identified in metazoans: Fic (also known as FicD and HYPE) localizes in the ER, and SelO localizes in the mitochondria (15, 16).

Using *Drosophila* as an animal model, we found that Fic is responsible for reversible AMPylation of BiP (10). During low ER stress or resting cells, Fic AMPylates BiP, thereby creating an inactive pool of BiP in the ER lumen (10, 17). When ER stress rises, BiP is deAMPylated and returned to an active state (10). Since this discovery, other laboratories have confirmed that this function is conserved in other metazoans, including *Caenorhabditis elegans*, rodents, and humans (16, 18, 19). We and others then demonstrated that Fic has dual catalytic activity for both the AMPylation and deAMPylation of metazoan BiP, and this activity changes depending on levels of ER stress (20, 21).

Further studies on the *Drosophila* model revealed that Fic plays a crucial role in protein homeostasis for metazoans. For Fic null flies (Fic<sup>−/−</sup>), the gross morphology of the fly eyes appeared normal, albeit they exhibited mild vision defects. When acute physiological ER stress was induced in fly eyes by exposure to continuous light, photoreceptors in wild-type flies, but not in Fic<sup>−/−</sup> mutants, could adapt (22). The damaged Fic<sup>−/−</sup> eyes exhibited severe structural defects in rhabdodemes (rhodopsin-containing membranes), elevated IRE1 activity, and reduced neurotransmission (22). Flies expressing Fic<sup>−/−</sup> that were unable to be AMPylated at Ser366 phenocopied the Fic<sup>−/−</sup> flies, with damaged rhabdodemes and loss of post synaptic responses for photoreceptors stressed with continuous light. Taken together, these studies support the proposal that having a reserve of inactive AMPylated BiP that can be immediately accessed by deAMPylation allows cells to deal with physiological stress more efficiently.

Overall, we propose Fic acts as a rheostat that tempers the cellular response to stress and maintains homeostasis by deAMPylation of modified BiP, thereby increasing levels of active BiP to alleviate mild ER stress. When the rheostat is disrupted, either by the absence of Fic or by a mutation in BiP that hampers its AMPylation, recovery from physiologically stressed cells is hindered, as there is no resource for immediate access to additional BiP pools. In the absence of this pool, more BiP can only be provided by the time-consuming transcription and translation of de novo BiP, coincidently with the triggering of UPR.

Based on these findings, we predicted that Fic is also required for the proper regulation of physiological stress in mammals. To address this hypothesis, we generated a conditional knockout line of Fic in the mouse. As with flies, the Fic<sup>−/−</sup> animals are viable, fertile, and appear healthy upon initial inspection. However, closer characterization of Fic<sup>−/−</sup> pancreata revealed altered responses to physiological and pathological stresses, with significant changes in UPR-induced signaling. We hypothesized that without Fic, the balance and threshold between the adaptive phase and the maladaptive phase of the UPR is shifted in tissues that rely heavily on ER secretory pathway to maintain protein homeostasis. Interestingly, we observe marked resilience in wild-type flies and mice when dealing with repeated stress. By contrast, both Fic<sup>−/−</sup> flies and Fic<sup>−/−</sup> mice lack the ability to efficiently recover from these stresses, resulting in damaged eyes and scarred pancreas, respectively. Taken together, our findings support the hypothesis that metazoan Fic plays a critical role by acting as a rheostat for the regulation of the UPR and protein homeostasis, likely to be important for the resilience of terminally differentiated, professional secretory cells that must respond to fluctuating needs of an organ.

**Results**

**Conditional deletion of Fic.** To determine the role of Fic-mediated AMPylation in the mammalian system, we chose to generate a conditional knockout line of Fic in the mouse. Using CRISPR-Cas9 technology, we constructed a floxed allele of Fic (Fic<sup>fl</sup>), in which two LoxP sites were integrated upstream of and within exon 3 (Fig. 1A). In addition, a single FLAG epitope was inserted into the C-terminal sequence of Fic’s coding sequence (Fig. 1A and SI Appendix, Fig. S1). Expression of Cre leads to Cre-Lox recombination that results in a nonfunctional Fic gene (Fic<sup>−/−</sup>) that is deleted for both the tetratricopeptide repeat (TPR) and Fic domains that are required for the targeting and AMPylation of BiP, respectively. The remaining truncated gene encodes only a small N-terminal peptide with the transmembrane sequence (Fig. 1A). Fic<sup>fl</sup> mice were bred to CAG-Cre transgenic mice (23) to generate Fic<sup>+/−</sup> mice, which were then backcrossed with Fic<sup>fl</sup> mice to generate Fic<sup>fl</sup>/fl mice. Germline transmission of this allele was confirmed through PCR and sequencing (SI Appendix, Fig. S2). Fic<sup>fl</sup>/fl mice were intercrossed to obtain sibling cohorts of Fic<sup>fl</sup>/fl and Fic<sup>−/−</sup> mice that were subsequently used in this study. Fic<sup>−/−</sup> mice were indistinguishable compared to Fic<sup>fl</sup>/fl and Fic<sup>fl</sup> littermates in viability, appearance, and weight (SI Appendix, Fig. S3).

**Fic<sup>−/−</sup> mice lack Fic protein, Fic messenger RNA, and BiP AMPylation.** Using qPCR analysis of liver complementary DNA, we confirmed loss of Fic transcript corresponding to exon 3 of Fic, validating the Fic knockout (Fig. 1B). Levels of BiP transcript were not significantly altered in Fic<sup>fl</sup>/fl and Fic<sup>−/−</sup> liver. We then attempted to validate deletion of Fic in mice using Western blot analysis of lysates from various tissues; however, due to the low expression level of endogenous protein, we were unable to detect Fic protein in wild-type or Fic<sup>fl</sup>/fl and Fic<sup>−/−</sup>/C0 mice that were subsequently used in this study. Fic<sup>−/−</sup> mice were distinguished by Fic<sup>fl</sup> and Fic<sup>fl</sup>/fl sections from Fic<sup>−/−</sup> mice, which were used for the genetic and messenger RNA expression data that Fic is not expressed in Fic<sup>−/−</sup> mice.

We next analyzed tissue for the presence or absence of Fic-mediated BiP AMPylation. Whole-cell lysates of livers from Fic<sup>fl</sup>/fl and Fic<sup>−/−</sup> littermates were analyzed for the presence of AMPylated BiP. Both Western blot analysis (Fig. 1D) and mass spectrometry analysis (SI Appendix, Fig. S5) indicate that BiP is no longer AMPylated in Fic<sup>−/−</sup> mice.

**Fasted Fic<sup>−/−</sup> mice display elevated serum amylase levels.** We predicted that Fic<sup>−/−</sup> mice would have dysfunction in tissues that rely heavily on UPR to maintain proteostasis. Although many tissues are known to require this regulation, we decided to focus on the initial study on Fic in the pancreas, a tissue that is well documented to rely on the UPR for proper exocrine and endocrine function (24, 25). Using the UPR fasting model, we screened Fic<sup>fl</sup>/fl and Fic<sup>−/−</sup> mice for serum markers that might indicate abnormalities in pancreatic function. A cohort of 10- to 11-week-old male mice were fasted overnight (∼14 h) with unrestricted access to water before sacrifice. Compared to Fic<sup>fl</sup>/fl and Fic<sup>fl</sup> controls, Fic null mice have normal weights (SI Appendix,
Fig. 1. Conditional deletion of Fic. (A) A schematic representation of wild-type and floxed allele of Fic (Fic<sup>fl/fl</sup>) in which LoxP sites were inserted into intron 2 and exon 3 of the Fic gene. A 6 amino acid FLAG sequence was added to the C terminus of the Fic ORF. (B) Quantification of Fic and BiP mRNA analyzed by qPCR from Fic<sup>fl/fl</sup> (blue bar) and Fic<sup>−/−</sup> (orange bar) mouse liver after 12 h fasting. n = 3. Bars indicate mean relative expression and error bars represent SE. Fic mRNA was below detection cutoff in Fic<sup>−/−</sup> samples. Statistics were performed using GraphPad Prism 9 using an unpaired t test. N.D., not detected; ns, not significant; **** < 0.0001. (C) Representative image of Fic and vimentin immunohistochemistry in coronal section of murine third ventricle. (Scale bar, 200 μm.) (D) Representative Western blot of liver lysates isolated from Fic<sup>fl/fl</sup> and Fic<sup>−/−</sup> mice. Blots were probed with anti-AMP, anti-BiP, and anti-actin antibodies. Asterisks (*) indicate reactivity to AMP antibody unrelated to Fic expression.

Fig. S3) and appear generally healthy. However, fasted serum levels of amylase were found to be significantly elevated in our Fic<sup>−/−</sup> cohorts (SI Appendix, Fig. S6). Of note, serum lipase levels and fasting glucose levels were not affected in our Fic<sup>fl/fl</sup> and Fic<sup>−/−</sup> mice. Since high amylase has been linked to hepatic dysfunction (26), we looked at another serum marker for hepatic dysfunction, aspartate aminotransferase (AST), to see if levels had changed, but levels of AST appeared normal in both Fic<sup>fl/fl</sup> and Fic<sup>−/−</sup> mice (SI Appendix, Fig. S6). Histopathological analysis of both the pancreas and liver revealed no detectable defect in either tissue (SI Appendix, Fig. S7).

Physiological stressed fasted-fed Fic<sup>−/−</sup> mice display altered UPR signaling in the pancreas. Based on the observed changes in serum amylase in our Fic<sup>−/−</sup> mice, we predicted that Fic<sup>−/−</sup> mice would show changes in UPR activation in the exocrine pancreatic tissue. To determine if this was the case, we utilized a mild physiological stress of fasting-feeding to activate the UPR in the exocrine pancreas. Fasting-feeding is a well-established method used to activate mild UPR in the pancreas. To determine if this was the case, we utilized the established method used to activate mild UPR in the pancreas. Fasting-feeding is a well-established model for pancreatic injury by injecting mice with caerulein, a cholecystokinin analog and secretagogue (29, 30). In this study, fasting-feeding (Fig. 3 A–D). Of note, no significant differences of these transcript levels were observed in the fasted-fed-recovery group, signifying that this difference in UPR induction is short lived.

Stressing Fic<sup>−/−</sup> mice with pancreatic caerulein treatment reveals changes in UPR response. Next, we wanted to assess the fitness of Fic<sup>−/−</sup> mice in response to a maladaptive, pathological ER stress and injury to the pancreas. For this, we used a well-established model for pancreatic injury by injecting mice with caerulein, a cholecystokinin analog and secretagogue (29, 30). In this study, pancreatitis was induced in 8- to 10-week-old female cohorts with seven hourly intraperitoneal (IP) injections of caerulein or, as a control, saline. Animals were sacrificed at 1, 4, 8, 24, and 72 h after the first injection (Fig. 4 A). Immediately
upon sacrifice, serum was collected from each mouse, and the pancreas was collected for RNA and histopathology.

As previously reported (29), treatment with caerulein induces acute pancreatitis. Analysis of serum revealed elevated levels of serum amylase and lipase were significantly altered, with reduced transcript levels for Chop and Hmox1 transcript levels also dropped significantly in Fic−/− mice compared to Ficfl/fl mice. Similarly, at 4 h after the first injection, transcript levels of Atf3 and Atf4 were comparable between Ficfl/fl and Fic−/− mice, whereas Hmox1 transcript levels were significantly lower in Fic−/− mice.

To assess if caerulein injury differentially alters the UPR response in Fic−/− mice compared to Ficfl/fl mice, we assessed the levels of UPR-induced transcripts in saline and caerulein-treated mice by qPCR. (SI Appendix, Fig. S11). As previously reported, UPR-induced transcripts are elevated upon caerulein treatment and resolve over the course of injury recovery (31–34). Whereas both Ficfl/fl and Fic−/− mice displayed similar levels of increased UPR transcript induction, the peak of transcript changes appeared earlier in Fic−/− mice.

One hour after the first injection of caerulein, levels of the UPR-induced transcripts Ask1, Egr1, and Fgf21 were found to be elevated in Fic−/− mice compared to wild-type mice (SI Appendix, Fig. S11 A–C). However, at 4 h after the first injection, Ask1 and Egr1 levels were comparable in wild-type and Fic−/− mice, and Fgf21 transcript levels were significantly lower than in wild type. Similarly, at 4 h after the first injection, levels of Atf3, Atf4, and Hmox1 transcripts were elevated in Fic−/− mice compared to Ficfl/fl (SI Appendix, Fig. S11 D–F). By 8 h after the first injection, transcript levels of Atf3 and Atf4 were comparable between Ficfl/fl and Fic−/− mice, whereas Hmox1 transcript levels were significantly lower in Fic−/− mice. BiP transcript levels also dropped significantly in Fic−/− mice at 8 h (SI Appendix, Fig. S11G). Expression patterns of Chop were also significantly altered, with reduced transcript levels for Fic−/− both at 4 and 8 h after the first caerulein injection (SI Appendix, Fig. S11H). Levels of Xbp1s appeared unaffected in Fic−/− mice (SI Appendix, Fig. S11I). Of note, the changes in Fic transcript levels in caerulein-treated mice mirrored the changes seen in Xbp1s transcript levels (SI Appendix, Fig. S11J). With the exception of Chop and Xbp1s, UPR transcripts appeared to peak and diminish in the pancreas of caerulein-treated Fic−/− mice early compared to Ficfl/fl mice.

**Fic is required for recovery of ER stress in both Drosophila and mice.** Next, we asked if Fic was required for cellular recovery after prolonged ER stress. In previous studies using...
Drosophila as a model, we found that fic⁻/⁻ flies were maladaptive to light stress. In this system, 3 d of constant light caused damage to ommatidial structures found in the fic⁻/⁻ compound eye and enhanced UPR activation (22). In these previous experiments, we found that the defects in rhabdomere integrity were completely reversible in wild-type flies, but, significantly, fic⁻/⁻ flies only partially recovered after 3 d of normal light/dark conditions.

As an extension of these previous experiments, we wanted to ask whether a repetitive version of this stress in flies results in a degenerative effect in affected tissues. To test this, we repeated treatments of constant light (LL) for 3 d, followed by 3 d of recovery (LD) on the same cohort of flies and scored for presence or lack of intact deep pseudopupils (DPPs), a visual indicator for disruption of ommatidial structure (Fig. 5A). As previously observed, for the first round of LL and LD treatment, wild-type flies completely recovered but fic⁻/⁻ flies showed only an 82% recovery. We found that with a second round of LL and LD treatment, wild-type flies retained their ability to fully recover but fic⁻/⁻ flies had only a 67% recovery. Finally, by the third repeat of this stress, wild-type flies continued to fully recover, whereas only 56% of fic⁻/⁻ flies retained intact DPPs.

Based on the changes in UPR response kinetics and increased serum amylase and lipase levels in caerulein-treated fic⁻/⁻ mice, we hypothesized that Fic⁻/⁻ mice might also exhibit more severe and prolonged histopathological changes in the pancreas upon caerulein-induced pancreatitis. The severity of pancreatitis in each saline- and caerulein-treated mouse was scored by severity of pancreatic edema, inflammatory infiltrate, and necrosis, as previously described (35). Based on these criteria, Fic⁺/⁺ and Fic⁻/⁻ mice exhibited comparable gross histological changes to the pancreas, with inflammation, edema, and necrosis observed in both cohorts over the course of the 72 h experiment (SI Appendix, Figs. S12 and S13 and Table S1), revealing no detectable difference in pancreatitis severity tissue after one round of acute caerulein injury.

Previous reports indicate wild-type mice typically fully recover from caerulein-induced acute pancreatitis by 7 d post-injury (36). We wanted to assess how Fic⁺/⁺ and Fic⁻/⁻ mice...

Fig. 3. UPR signaling in fasted-fed Fic⁺/⁺ and Fic⁻/⁻ pancreata. (A–F) Quantification of Bip, Atf4, Xbp1s, Chop, Atf3, and Fgf21 mRNA analyzed by qPCR from Fic⁺/⁺ (blue bar) and Fic⁻/⁻ (orange bar) mouse pancreas after fasting, fast-feeding, and fast-feed-recovery. Expression values were normalized to that of the housekeeping gene U36B4. Bars indicate mean relative expression compared to fasted controls, and error bars represent SE. Statistics were performed using GraphPad Prism 9 using a two-way ANOVA. n = 8. *P < 0.05; **P < 0.01.

Fig. 4. Caerulein-induced pancreatitis in Fic⁺/⁺ and Fic⁻/⁻ mice. (A) A schematic representation of caerulein-induced acute pancreatitis induction. (B, C) Quantification of serum amylase and lipase in Fic⁺/⁺ and Fic⁻/⁻ mice over 72 h of caerulein-induced acute pancreatitis. Bars indicate mean and error bars represent SE. Statistics were performed using GraphPad Prism 9 using an two-way ANOVA. n = 5–7. ****P < 0.0001.

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recovered from caerulein-induced pancreatitis. A cohort of 6- to 8-week-old \textit{Fic}\textsuperscript{+/+} and \textit{Fic}\textsuperscript{−/−} male mice was induced for pancreatitis with seven hourly IP injections of saline or caerulein and left to recover for 7 d. Mice were then split into two groups: a fasted and fasted-fed group as previously described (Fig. 2A). Immediately upon sacrifice, RNA and tissue for histopathology from the pancreas of each mouse were collected.

As previously described (Fig. 2B), \textit{Fic}\textsuperscript{−/−} mice had elevated serum amylose levels upon fasting and fasting-feeding, but the elevation of serum amylose was no longer affected, indicating that both \textit{Fic}\textsuperscript{+/+} and \textit{Fic}\textsuperscript{−/−} mice had recovered from the initial caerulein-induced injury (Fig. 5B). Notably, serum lipase of caerulein-treated \textit{Fic}\textsuperscript{−/−} mice after fast-feeding was elevated. Moreover, histopathological scoring of the pancreas showed an increased incidence of fibrosis in caerulein-treated \textit{Fic}\textsuperscript{−/−} mice than \textit{Fic}\textsuperscript{+/+} after 7 d of recovery (Fig. 5 C and D and Table 1). These data suggest that \textit{Fic}\textsuperscript{−/−} mice have increased scarring and reduced capacity for recovery from tissue damage.

**Discussion**

The UPR is crucial for the maintenance of protein homeostasis during physiological stress of cells with high secretory capacity. When cellular stress levels reach maladaptive levels, recovery from stress becomes challenging due to prolonged attenuated protein synthesis of nonstress-responsive genes and activation of apoptotic machinery. Thus, regulation of the UPR must be tightly coordinated to meet the needs of the tissue. To

**Table 1. Histopathological scoring of caerulein pancreatitis after 1 wk recovery**

| Genotype | Treatment | Edema | Inflammation | Necrosis | Fibrosis | Total |
|----------|-----------|-------|--------------|----------|----------|-------|
| \textit{Fic}\textsuperscript{+/+} | Saline | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| \textit{Fic}\textsuperscript{+/+} | Caerulein | 0 ± 0 | 0.13 ± 0.085 | 0 ± 0 | 0.31 ± 0.18 | 0.44 ± 0.24 |
| \textit{Fic}\textsuperscript{−/−} | Saline | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| \textit{Fic}\textsuperscript{−/−} | Caerulein | 0.08 ± 0.077 | 0.39 ± 0.18 | 0.077 ± 0.077 | 0.92 ± 0.25 | 1.46 ± 0.48 |

Average scores ± SEM.
understand the role of reversible AMPylation of BiP in protein homeostasis in a mammalian system, we generated a conditional knockout of Fic in mice. We speculated that tissues reliant on secretion might be most affected by the deletion of Fic and therefore focused our initial efforts on the pancreas. Using both a fast-feeding and a caerulein-induced pancreatic injury model, we observed changes to UPR signaling and physiology of the pancreas suggestive of exocrine pancreas dysfunction in Fic−/− mice. Analysis of UPR markers for these experiments revealed changes in the timing and duration of the UPR transcriptional response (Fig. 3 and SI Appendix, Fig. S11).

Furthermore, analysis of tissue recovery after light-induced or caerulein-induced damage in Drosophila eyes and mouse pancreas, respectively, indicates that loss of Fic reduces recovery from ER stress-associated tissue damage in both animal models (Fig. 5). Therefore, the loss of Fic-mediated AMPylated BiP leaves tissues vulnerable to irreversible damage with chronic and repeated stresses.

Our and other groups have proposed that inactivating PTMs on BiP could provide a mechanism by which rapidly changing physiological fluctuations of the ER stress can be nimbly regulated (37). AMPylation of BiP by Fic allows for a pool of inactive chaperone to remain in the ER without deleterious consequences to protein folding that might otherwise be hindered in the presence of excess chaperone (37, 38). Previous studies indicate that the pool of inactivated BiP is significant in various cell types, ~40% in fasted pancreas and over 50% in unstressed Drosophila S2 cells (10, 37). This inactive pool of BiP can then be readily activated to address increasing loads of unfolded proteins in cells with rapidly fluctuating demands on protein synthesis and secretion, such as the pancreas, while tempering the activation of the UPR.

We predict that Fic provides a necessary level of regulation of the UPR to properly adjust protein homeostasis in tissue with frequent physiological ER stress (Fig. 6). By keeping a readily accessible pool of inactive BiP, cells can provide a nimble response to ER stress through a short burst of UPR activation. Rapid deAMPylation of BiP results in additional active chaperone much faster than what can be accomplished by new protein synthesis. This results in smaller, more moderate pulses of UPR signaling under repeated physiological stresses, keeping protein synthesis and secretion, such as the pancreas, while tempering the activation of the UPR.

Cells without Fic regulation of BiP lack this pool of chaperone on standby, resulting in prolonged and elevated UPR signaling, as a delayed response requires more chaperone via transcription and translation to accommodate the increased physiological stress. This is supported by our qPCR analysis of UPR-responsive genes in Fic−/− mice under both physiological and pharmacological stresses (Fig. 3 and SI Appendix, Fig. S11). Repetitive stress in Fic−/− tissues would result in amplified UPR, leading to progression into the maladaptive phase of the UPR and tissue damage over the lifetime of the tissue. Our data point to evidence of this in the exocrine pancreas, where elevated UPR signaling and serum amylase indicate functional disruption. As elevated serum amylase is one of the first key clinical indicators of pancreas dysfunction, we suspect additional and continued physiological stresses to Fic−/− tissue will lead to increased prevalence of disease.

The pancreas primarily comprises terminally differentiated professional secretory cells with limited regenerative capability. Therefore, the pancreas must employ mechanisms to ensure resilience to repetitive stress in order to last and properly function for the lifetime of the animal. Herein, we provide evidence that Fic provides one such mechanism through the moderation of the UPR during physiological stress. Similarly, a wild-type fly eye has the capacity to recover from the physiological stress of continuous light. In the absence of the Fic rheostat, the fic−/− eyes are challenged over time and lose the potential to regenerate rhabdomere integrity. Analogously, we observe more scarring in the injured Fic−/− pancreas.

Many studies to date have used tissue culture cell lines as a model to study the UPR in which a chemical stress is applied to cells resulting in a very strong, and frequently irreversible, induction of the UPR (39). Under these conditions, tissue culture cells respond in basically two ways, cell death or replication, allowing for new cells to overcome the stress. These options are far from optimal for differentiated cells within a tissue where cellular function needs to be maintained for survival of the organ and/or animal. We predict that many subtleties of UPR regulation will only be apparent under such physiological stresses in the context of specific tissues. Thus, it is not surprising that studies with tissue culture models have only exhibited very subtle differences in activation of UPR in the absence of Fic (19). Systems in an animal that use cells with high regenerative capacity and shorter life spans may not require Fic mediation of the UPR, as turnover and replenishment with new cells will bypass the need of rheostat. This is consistent with observations by other groups with a Fic deletion model (40). In sum, we predict that terminally differentiated postmitotic cells will be principally reliant upon the Fic-mediated rheostat to maintain a healthy response to continuing physiological stress over an animal’s lifetime.

Whereas this study focuses on this one tissue only, we speculate that other tissues with professional secretory, terminally differentiated cells that must adapt to fluctuating stress will be similarly affected in the Fic−/− mouse. We propose the presence of Fic rheostat allows for tempering of the UPR response by maintaining a window for reversible UPR response that is critical for maintenance of protein homeostasis. The importance of this window has been highlighted with the treatment of UPR stress with the pharmacological agent ISRIB (integrated stress response inhibitor) where it is only observed to be efficacious during the adaptive phase of UPR (9). Future studies with ISRIB and Fic−/− mice will be useful for understanding the importance of the Fic-mediated rheostat and treatment of disease.

For the health of an animal, it is critical to maintain resilience in terminally differentiated cells during repeated physiological stress to prevent disease. We predict that Fic regulation of the UPR will play a role in mitigating the deleterious effects of UPR activation in a variety of tissues with UPR-associated diseases.
including retinal degeneration, atherosclerosis, metabolic syndrome, and various neurodegenerative disorders. Our future studies will focus on the identification of tissues in which Fic plays a role in the regulation of the UPR and the physiological consequences of the absence of Fic-mediated regulation of the UPR.

Materials and Methods

Detailed descriptions of the experimental methods are provided in SI Appendix, SI Materials and Methods. These include mouse protocols, generation of conditional Fic knockout, histology, quantitative real-time PCR, immunofluorescence of brain sections, mass spectrometry analysis, Western blot analysis, and deep pseudopod analysis.

Data Availability. All study data are included in the article and/or supporting information.

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