Fortilin Binds IRE1α and Prevents ER Stress from Signaling Apoptotic Cell Death

Matthew D. King
Boise State University

Owen M. McDougal
Boise State University

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Fortilin binds IRE1α and prevents ER stress from signaling apoptotic cell death

Decha Pinkaew1, Abhijnan Chattopadhyay1,2, Matthew D. King3, Preedakorn Chunhacha1, Zhihe Liu1, Heather L. Stevenson4,5, Yanjie Chen1, Patuma Sinthujaroen1, Owen M. McDougal3 & Ken Fujise1,2,5

The endoplasmic reticulum, the cytoplasmic organelle that matures a massive amount of nascent secretory polypeptides, is particularly sensitive to stress. Endoplasmic reticulum stress causes unfolded proteins to populate the organelle, eliciting the unfolded protein response. During the unfolded protein response, GRP78—a master endoplasmic reticulum stress regulator—detaches from three endoplasmic reticulum stress sensors (IRE1α, PERK, and ATF6) and allows them to activate the apoptotic signaling pathway. Fortilin, a pro-survival molecule, is known to inhibit apoptosis by binding and inhibiting p53, but its role in endoplasmic reticulum stress-induced apoptosis remains unknown. Here, we report that fortilin directly interacts with the cytoplasmic domain of IRE1α, inhibits both kinase and endoribonuclease (RNase) activities of the stress sensor, and protects cells against apoptotic cell death at both cellular and whole animal levels. Our data support a role of fortilin in the unfolded protein response and its potential participation in human diseases caused by unfolded protein response.
Precipitated by nutrient deprivation, hypoxia, and reactive oxygen species, endoplasmic reticulum (ER) stress causes protein folding to slow and unfolded proteins to accumulate in the organelle, eliciting the unfolded protein response (UPR). The UPR is a cellular process highly conserved across species that is designed to restore and enhance the ability of the ER to fold and process proteins and to avoid the catastrophic outcome (i.e., death of the organism) of uncontrolled and overwhelming accumulation of misfolded proteins. During the UPR, GRP78 (also known as BiP)—an ER resident master stress regulator protein—detaches from three key ER transmembrane stress sensors (IRE1, PERK, and ATF6) to bind and sequester defective proteins. When freed from the binding and suppression of GRP78, IRE1, PERK, and ATF6 become activated and initiate the UPR.

ER stress translocates fortilin from nucleus to cytosol. To test whether fortilin changes its intracellular localization upon ER stress, we stimulated the PC3 human prostate cancer cell line with either thapsigargin (TG) or the epidermal growth factor (EGF) fused to the proteolytic A subunit of a bacterial AB5 toxin (SubA) (EGF-SubA), subjected cells to subcellular fractionation, and quantified fortilin concentrations in the nuclear, cytosolic, and ER fractions using immunoblot analysis. TG is a well-characterized ER stress-inducing agent that induces ER stress in the cell by binding to and inhibiting Ca2+-ATPase, an ER resident transmembrane protein that maintains Ca2+ homeostasis. EGFR-SubA is an engineered fusion protein. When exposed to EGFR-SubA, cells expressing the EGF receptor internalize the fusion molecule into the cytosol. EGF-SubA is then retrogradely transported via the Golgi system to the ER lumen, where it selectively and rapidly cleaves and destroys GRP78. Fortilin is the only known substrate of SubA, EGFR-SubA represents a highly specific inducer of ER stress. At the baseline, fortilin was present in all three fractions (Fig. 1a, from c1 to c2; from c3 to c4). Together, these data suggest that ER stress translocates fortilin from the nucleus to the ER region of the cytosol.

Fortilin protects cells against ER-stress-induced apoptosis. To test whether fortilin can protect cells against ER stress-induced apoptosis, we generated PC3 cells lacking fortilin (PC3sh_Fortilin) by lentivirally introducing short-hairpin-RNA against fortilin (sh-Fortilin) into the cell. PC3sh_Fortilin expressed significantly less fortilin than did the control cells (PC3sh_Empty) (Fig. 1b; Supplementary Fig. 6). Next, we challenged these cells with either TG (Fig. 1c) or EGF-SubA (Fig. 1d) and quantified the degree of DNA fragmentation. Both TG and EGF-SubA dose-dependently induced DNA fragmentation in both PC3sh_Empty and PC3sh_Fortilin cells (Fig. 1c, d). However, PC3sh_Empty cells exhibited significantly less DNA fragmentation than did PC3sh_Fortilin cells at all concentrations of TG and EGF-SubA (Fig. 1c, d). These data indicate that fortilin protected PC3 cells against ER stress-induced apoptosis. Next, we generated U2OS cells overexpressing fortlin (U2OSFortilin_HA) and control cells (U2OSEmpty_HA) (Supplementary Figs. 1B and 9), challenged the cells with TG, and assessed the survival (Supplementary Fig. 1C) and degree of DNA fragmentation (Supplementary Fig. 1D) of these cells. Fortilin protected the U2OS cells against TG-induced cell death and DNA fragmentation (Supplementary Fig. 1C, D).

To evaluate how fortilin modulates ER stress-induced apoptosis and necrosis, we challenged PC3sh_Fortilin and PC3sh_Empty cells with either TG or EGF-SubA, stained them with fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide (PI), and analyzed them using flow cytometry. Upon TG and EGF-SubA challenge, PC3sh_Fortilin cells bound more Annexin V than did PC3sh_Empty cells (Fig. 1e, Apoptosis; *P < 0.05 by two-tailed unpaired t-test), although there was no difference in PI levels between the two groups of cells (Fig. 1e, Necrosis; NS = not statistically significant). These data suggest that fortilin protected PC3 cells against ER stress-induced apoptosis but not against necrosis (Fig. 1e).

To assess the role of fortilin in the ER stress-induced activation of caspases, we challenged PC3sh_Fortilin and PC3sh_Empty Cells with either TG or EGF-SubA, and subjected the cell lysates to immunoblot analysis (Fig. 1f, g; Supplementary Fig. 6). Both TG and EGF-SubA induced more cleavage of caspases-9 and -3.
PC3sh-Empty cells were challenged by either TG or EGF-SubA and subjected to the DNA fragmentation assay to evaluate the degree of apoptosis. 

Fractions were evaluated by IB.

Next, we challenged PC3sh-Fortilin and PC3sh-Empty cells with TG and quantified the activities of caspase-3, -8, and -9. TG dose-dependently activated caspase-9 and -3 but not -8, as previously reported. In this system, fortifin protected TG-challenged U2OS cells against the activation of caspases-9 and -3 (Supplementary Fig. 1E). In the top panel, PC3sh-Fortilin and PC3sh-Empty cells were challenged by either TG or EGF-SubA, fractionated the lysates into cytosolic and mitochondrial fractions, and subjected them to immunoblot analysis using anti-cytochrome c antibody. The cytosolic fraction of PC3sh-Fortilin cells contained a greater amount of cytochrome c upon TG/EGF-SubA stimulation than the control (Fig. 1h; c vs. c4 for TG; f3 vs. f4 for EGF-SubA; Supplementary Fig. 7). The mitochondrial fraction of PC3sh-Fortilin cells contained a lesser amount of cytochrome c upon TG/EGF-SubA stimulation than the baseline (Fig. 1h; c7 vs. c8 for TG; f7 vs. f8 for EGF-SubA). TG/EGF-SubA stimulation did not change the amount of cytochrome c in the cytosol or mitochondria in PC3sh-Empty cells (Fig. 1h; c1 vs. c2, c5 vs. c6 for TG; f1 vs. f2, f5 vs. f6 for EGF-SubA). These data indicate that fortifin prevents cytochrome c release from mitochondria to the cytosol in TG/EGF-SubA-stimulated PC3 cells.

Fig. 1 Fortifin protects cells against apoptosis under ER stress. 

RG and PARP in PC3sh-Fortilin than in PC3sh-Empty cells (Fig. 1f for TG: caspase-9, c2 and c3 vs. c5 and c6; caspase-3, e2 and e3 vs. e5 and e6; PARP, g2 and g3 vs. g5 and g6, respectively) (Fig. 1g for EGF-SubA: caspase-9, c2 and c3 vs. c5 and c6; caspase-3, e2 and e3 vs. e5 and e6; PARP, g2 and g3 vs. g5 and g6, respectively).

Next, we challenged U2OS Empty-HA and U2OS Fortilin-HA cells with TG and quantified the activities of caspase-3, -8, and -9. TG dose-dependently activated caspase-9 and -3 but not -8, as previously reported. In this system, fortifin protected TG-challenged U2OS cells against the activation of caspases-9 and -3 (Supplementary Fig. 1E). In the top panel, PC3sh-Fortilin and PC3sh-Empty cells were challenged by either TG or EGF-SubA, fractionated the lysates into cytosolic and mitochondrial fractions, and subjected them to immunoblot analysis using anti-cytochrome c antibody. The cytosolic fraction of PC3sh-Fortilin cells contained a greater amount of cytochrome c upon TG/EGF-SubA stimulation than the control (Fig. 1h; c vs. c4 for TG; f3 vs. f4 for EGF-SubA; Supplementary Fig. 7). The mitochondrial fraction of PC3sh-Fortilin cells contained a lesser amount of cytochrome c upon TG/EGF-SubA stimulation than the baseline (Fig. 1h; c7 vs. c8 for TG; f7 vs. f8 for EGF-SubA). TG/EGF-SubA stimulation did not change the amount of cytochrome c in the cytosol or mitochondria in PC3sh-Empty cells (Fig. 1h; c1 vs. c2, c5 vs. c6 for TG; f1 vs. f2, f5 vs. f6 for EGF-SubA). These data indicate that fortifin prevents cytochrome c release from mitochondria to the cytosol in TG/EGF-SubA-stimulated PC3 cells.

Fortifin protects cells against apoptosis, but not necrosis, under TG- and EGF-SubA-induced ER stress. 

Fortifin protects cells against ER stress-induced caspase-3, -9, and PARP. PC3sh-Fortilin and PC3sh-Empty cells were challenged by either TG or EGF-SubA, stained with Annexin V and Calreticulin to evaluate the role of fortilin in the prevention of apoptosis and necrosis. Data were expressed as means ± s.d. (biological replicates [n] = 3) and analyzed by two-tailed unpaired t-test. NS not statistically significant; *P < 0.05; ***P < 0.005.
Fortilin prevents ER stress from activating IRE1α. EGF-SubA targets a single protein, namely GRP78, in mammalian cells. Cells expressing a SubA-resistant mutant GRP78 (GRP78L416D) are immune to EGF-SubA, indicating that EGF-SubA is a selective ER stress-inducing agent with minimum off-target effects. To further study the role of fortin in ER stress signaling in a system free of potential downstream effects of TG-induced perturbations in Ca2+, we performed all remaining experiments using EGF-SubA. To confirm that EGF-SubA cleaves GRP78 in PC3 cells, we incubated PC3 cells with 0–20 nM of EGF-SubA and subjected the lysates to immunoblot analysis using anti-GRP78 antibody. EGF-SubA dose-dependently cleaves GRP78 in PC3 cells (Fig. 2a; Supplementary Fig. 7), but treatment with EGF-SubA did not affect the expression levels of cleaved GRP78 in PC3 cells (Fig. 2a; Supplementary Fig. 7). Both PC3sh-Fortilin and PC3sh-Empty cells expressed similar amounts of IRE1α, PERK, and ATF6 (both p54 and p46), regardless of the concentrations of EGF-SubA and subjected to immunoblot analysis (Fig. 2d, e; Supplementary Fig. 7). Both PC3sh-Fortilin and PC3sh-Empty cells expressed similar amounts of IRE1α and JNK (both p54 and p46), regardless of the concentrations of EGF-SubA (Fig. 2e, rows c and e of lanes 1–4 vs. lanes 5–8, and the bottom graph). Strikingly, however, PC3sh-Fortilin cells expressed greater amounts of phosphorylated and activated forms of IRE1α (P-IRE1α) and JNK (P-JNK) than did PC3sh-Empty cells (Fig. 2e, rows b and d of lanes 2–4 vs. lanes 6–8). Enzyme-linked immunosorbent assay (ELISA) analysis of the cell lysates from PC3sh-Fortilin and PC3sh-Empty cells treated with various concentrations of EGF-SubA revealed a trend that supports these results (Fig. 2f).
concentrations of EGF-SubA also showed that fortilin protected PC3 cells against the phosphorylation of JNK (Supplementary Fig. 2). Furthermore, the protein expression and mRNA levels of XBP1— the active form of XBP1 generated by P-IRE1α— were greater in PC3_h-Fortilin than in PC3_h-Empty cells (Fig. 2e, row f of lanes 2–4 vs. lanes 6–8 and Fig. 2f, lanes 2–4 vs. lanes 6–8, respectively, and the bottom graphs). Consistent with previous reports that ATF6 does not participate in ER stress-induced apoptosis,

![Diagram](image1)

**Fig. 3** Fortilin interacts with the cytosolic domain of IRE1α and inhibits its protein kinase and RNase activities. a Proximity ligation assay (PLA) shows a specific interaction between fortilin and P-IRE1α in EGF-SubA-treated PC3 cells. The cells were treated with 2 nM EGF-SubA for 24 h and subjected to PLA, using anti-IRE1α and anti-P-IRE1α antibodies to evaluate fortilin-IRE1α and fortilin-P-IRE1α interaction, respectively. PLA interaction indices were calculated by dividing the number of red dots by the number of nuclei, expressed as means ± s.d. (n = 3), and analyzed by two-tailed unpaired t-test. NS not statistically significant; ***P < 0.005. Scale bar = 10 μm. b Fortilin co-immunoprecipitates P-IRE1α. PC3 cells were treated with 2 nM EGF-SubA for 24 h, lysed and subjected to immunoprecipitation (IP). c Domain structure of human IRE1α. Human IRE1α consists of the ER luminal domain (aa 1–443), transmembrane domain (aa 444–464), linker region (aa 465–567), kinase domain (aa 568–833), and endoribonuclease (RNase) domain (aa 836–997). The following recombinant proteins were used for biolayer interferometry: full-length IRE1α (aa 1–977), IRE1α-Myc-DDK (aa 1–977); IRE1α-L, GST-IRE1α (aa 1–70); IRE1α-TM, GST-IRE1α (aa 401–500); and IRE1α-C, GST-IRE1α (aa 468–977). d–h Fortilin binds to P-IRE1α through its cytosolic domain. Biotinylated fortilin was immobilized to the streptavidin biosensor. Recombinant IRE1α, either full-length or fragment, was applied to the biosensor at various concentrations, and dissociation constants (Kd, expressed as mean ± s.d., n = 3) were derived. i Lowest energy binding pose of fortilin (blue) with cytosolic domain of IRE1α (green) (the right panel) presented with that of a fortilin-fortilin dimer (the left panel). j Intermolecular interactions between phosphorylated serine724 (pS724) and serine726 (pS726) of the cytosolic domain of IRE1α with lysine residues (K19 and K34) of fortilin. k Fortilin inhibits the RNase activity of IRE1α. An in vitro IRE1α RNase assay was performed by incubating IRE1α with human recombinant fortilin and the substrate fluorescently tagged XBP1 RNA stem loop, the cleavage of which would allow the fluorescein amidite (FAM) to fluoresce. Data were expressed as means ± s.d. (n = 4) and analyzed by two-tailed unpaired t-test. ***P < 0.005. l Fortilin inhibits the kinase activity of IRE1α. An in vitro IRE1α kinase activity assay was performed by incubating IRE1α with [γ-33P]ATP, recombinant fortilin, and myelin basic protein (MBP) as a substrate of the kinase in the kinase reaction buffer. The phosphorylation index was calculated by dividing the radioactivity of MBP for a given fortilin concentration by that of the vehicle treatment. The phosphorylation index was calculated by dividing the number of red dots by the number of nuclei, expressed as means ± s.d. (n = 3), and analyzed by two-tailed unpaired t-test. NS not statistically significant; ***P < 0.005. Scale bar = 10 μm.
Fortilin binds the cytosolic domain of IRE1α. To investigate how fortilin selectively and negatively regulates IRE1α and its signaling pathway, we tested whether fortilin binds IRE1α using the in situ proximity ligation assay (PLA)

We counted the number of red dots and divided it by the number of nuclei to calculate the PLA interaction index. EGFR-SubA-induced ER stress drastically increased the number of interactions between fortilin and P-IRE1α (Fig. 3a, right panel, bars 3 vs. 4) but not the number of interactions between fortilin and IRE1α (Fig. 3a, right panel, bars 1 vs. 2).

To further evaluate the interaction between fortilin and P-IRE1α in vivo, we challenged PC3 cells with 2 nM EGFR-SubA for 24 h and incubated the cleared total cell lysates with either rabbit anti-fortilin monoclonal antibody (Clone EPR5540) or rabbit IgG. We precipitated the formed protein complex using Protein G (Dynal-ThermoFisher Scientific, Waltham, MA, USA), washed them extensively, eluted them into the sodium dodecyl sulfate (SDS) loading buffer, and subjected them to SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting. The rabbit anti-fortilin antibody, but not rabbit IgG, successfully immunoprecipitated fortilin (Fig. 3b, band a2; Supplementary Fig. 7). Both β-actin and α-tubulin—known fortin binding proteins[32, 33]—were co-immunoprecipitated with fortilin (Fig. 3b, band c2 and f2).

NQO2—a protein known not to interact with fortifin[34]—was not co-immunoprecipitated, suggesting that the wash condition was sufficiently stringent (Fig. 3b, no band in d2). In this system, both anti-P-IRE1α and anti-IRE1α antibodies detected discrete bands at the expected molecular weights (Fig. 3b, bands b2 and c2, respectively). The immunoblot signals of fortifin, P-IRE1α, IRE1α, β-actin, and α-tubulin (Fig. 3b, bands a2, b2, c2, e2, and f2, respectively) were from specific protein–protein interactions and did not represent non-specific interaction between the proteins and the beads because there were no immunoblot signals in the sample incubated with the same beads and IgG (Fig. 3b, a3, b3, c3, d3, e3, and f3). Because anti-IRE1α antibody is known to detect both non-phosphorylated and phosphorylated IRE1α, band c2 (Fig. 3b) could be either non-phosphorylated or phosphorylated IRE1α. Importantly, however, the presence of a band in the membrane probed by anti-P-IRE1α (Fig. 3b, band b2) shows the presence of a specific interaction between fortifin and P-IRE1α. We found the same in PC3 cells treated by TG (Supplementary Figs. 3A and 9). These data, when taken together, suggest that fortifin specifically interacts with P-IRE1α.

IRE1α is a transmembrane protein consisting of five distinct domains—luminal, transmembrane, linker, kinase, and RNase domains[35] (Fig. 3c). To further characterize the direct physical interaction between fortifin and P-IRE1α, we tested if biolayer interferometry (BLItz, ForteBio, Menlo Park, CA, USA)[36] could be used to detect the interaction between fortifin and its binding partners. We first biotinylated and immobilized recombinant human fortifin (Supplementary Fig. 3B, lane 9) to the streptavidin-coated biosensor. BLItz appropriately detected the interaction between fortifin on the biosensor and anti-fortilin monoclonal antibody in the aqueous phase at the dissociation constant (Kd) of 71.46 ± 0.04 nM (mean values ± s.d., n = 3, Supplementary Fig. 3C), whereas no meaningful interaction between fortifin and albumin was detected (Kd = 1.05 ± 0.11 mM, mean values ± s.d. n = 3, Supplementary Fig. 3D). Next, we subjected human recombinant fortifin and IRE1α and its deletion mutants to the same biolayer interferometry (BLItz)[36]. Five different recombinant preparations of IRE1α (dephosphorylated IRE1α, 1–977 aa, see Supplementary Figs. 3E and 9), P-IRE1α (phosphorylated IRE1α, 1–977 aa), IRE1α-L (the N-terminal, luminal portion of IRE1α, 1–70 aa), IRE1α-TM (the transmembrane portion of IRE1α, 401–500 aa), and IRE1α-C (the cytosolic portion of IRE1α, 468–977 aa) (Fig. 3c and Supplementary Fig. 3B, lanes 2, 5, 6, and 7). Fortilin bound only weakly to IRE1α (Fig. 3d) but tightly to P-IRE1α (Fig. 3e). Further, fortilin did not bind the luminal or transmembrane portions of IRE1α (Fig. 3f, g) but bound the cytosolic portion of IRE1α (Fig. 3h). These data suggest that fortifin specifically and directly binds to the activated and phosphorylated form of IRE1α (P-IRE1α) through its cytosolic domain (Fig. 3c–h and Supplementary Fig. 3G).

To further verify the binding of fortifin to IRE1α, we performed molecular docking and molecular dynamics (MD) experiments using the methods described in detail in the Methods section. Because fortifin has been shown to dimerize[37], we performed a molecular docking study for both dimerized fortins (fortilin:fortilin) and the fortin–IRE1α-C complex (fortin: IRE1α-C). The binding surface of fortifin that is used to interact with another fortin for dimerization (Fig. 3i, the left panel) was nearly identical to that used to interact with IRE1α-C (Fig. 3i, the right panel). The relative binding favorability scores for the fortin:fortin and fortin:IRE1α-C complexes were calculated to be −879.1 and −945.3 kcal mol⁻¹, respectively, suggesting that the fortin:IRE1α-C complex was more thermodynamically stable than the fortin:fortin complex. We then inspected the docking pose of the fortin:IRE1α-C complex and found that the main phosphorylation sites (S²⁷⁴ and S²⁷⁶) of IRE1α[38] were positioned in close proximity to the binding surface of fortin (especially K¹⁹ and K¹⁹) regardless of their phosphorylation status, suggesting that the binding of fortifin to IRE1α blocks the access of anther IRE1α to the phosphorylation sites (S²⁷⁴ and S²⁷⁶) of the molecule for trans-autophosphorylation. The adenosine diphosphate (ADP) binding region of the kinase domain (R⁶⁰⁰, E⁶¹₂, D⁶⁸⁸, N⁶⁹³, and D⁷¹¹)[38] and the RNA processing region of the RNase domain (Y⁸⁹², R⁹⁰⁴, N⁹⁰⁵, and H⁹⁰⁹)[38] were not positioned near the binding surface of fortifin, although it is likely that binding of fortifin to the key phosphorylation sites of IRE1α induces conformational changes to disrupt the functionality of these regions[38].

Next, we compared the thermodynamic stability of the fortin–P-IRE1α complex with that of the fortin–IRE1α complex using MD forward and reverse binding free energy simulations. We found that the fortin–P-IRE1α complex was substantially more thermodynamically stable than the fortin–IRE1α complex by a ΔGbind value of −15.48 kcal mol⁻¹ (Supplementary Table 1). The free energy change (ΔG) due to phosphorylation of IRE1α in the bound state with fortin was −602.61 ± 1.02 kcal mol⁻¹ (mean values ± s.e., whereas the free energy change (ΔG) due to phosphorylation of unbound IRE1α was −587.13 ± 1.01 kcal mol⁻¹ (mean values ± s.e.). Both the forward and reverse simulations, which correspond to phosphorylation and dephosphorylation of S²⁷⁴ and S²⁷⁶ of IRE1α, resulted in comparable values, thus supporting the validity of the calculations. The higher thermodynamic stability of the fortin–P-IRE1α complex compared to that of fortin–IRE1α may be due to the strong electrostatic forces between the positively charged lysine residues (K¹⁹ and K¹⁹) of fortin and the negative charges of the phosphoserine residues (S²⁷⁴ and S²⁷⁶) (Fig. 3j). These MD data suggest that fortifin binds IRE1α in a thermodynamically stable fashion, that fortifin binds preferentially to P-IRE1α over IRE1α, and that fortin binding to P-IRE1α occludes the key phosphorylation sites of IRE1α.
sites (S724 and S726) of the molecule and thus can prevent IRE1α dimerization and subsequent trans-autophosphorylation.

Fortilin inhibits both kinase and RNase activities of IRE1α.

The cytosolic domain of IRE1α contains the kinase and RNase domains (Fig. 3c). Because fortilin prevented EGF-SubA from phosphorylating IRE1α (row b of Fig. 2e and Supplementary Fig. 2) and splicing XBP1 to form XBP1s (Fig. 2f and row f of Fig. 2e), we tested whether fortilin inhibits the kinase and RNase activities of IRE1α through its direct interaction with the ER stress sensor molecule. We first assessed the RNase activity of IRE1α in the presence of fortilin in vitro using a synthetic mRNA stem loop corresponding to the XBP1 substrate sequence39. The
Fortilin saves mice from ER stress-induced liver failure. To study how protection by fortin might compensate for the action of the IRE1α pathway manifests itself in the whole animal under ER stress, we generated a mouse strain that lacks fortin specifically in the liver—alb-Cre<sup>−/−</sup>fortin<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup>liver<sup>KO-liver</sup>—by crossing transgenic mice overexpressing the albumin-promoter-driven Cre recombinase (alb-Cre<sup>−/−</sup>) with fortin<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup>liver<sup>KO-liver</sup> (Supplementary Fig. 4A, B, C). These mice appeared normal and were fertile. The liver of the fortin<sup>KO-liver</sup> mice expressed very little fortin (Fig. 4a, top panel; Supplementary Fig. 8), whereas fortin expression was similar in other organs between fortin<sup>KO-liver</sup> and fortin<sup>WT-liver</sup> mice (Fig. 4a, bottom panel). We then intraperitoneally administered a one-time dose of EGFR-SUBA (250 µg kg<sup>−1</sup> body weight) to fortin<sup>KO-liver</sup> and fortin<sup>WT-liver</sup> mice. The liver expresses the EGF receptor and thus responds to EGFR-SUBA<sup>43</sup>. The first group of animals (<i>n = 10</i> per group) were observed for 48 days to assess survival (Fig. 4b, the left arm, “Survival”), whereas the animals in the second group were sacrificed on day 7, and the blood and organs were harvested for further analyses (Fig. 4b, the right arm, the mechanism of action or “MOA”). In the survival experiment, 80% of fortin<sup>WT-liver</sup> mice were still alive on day 48, whereas none of the fortin<sup>KO-liver</sup> mice survived (all died between days 9 and 12) (<i>P < 0.001</i> by the Kaplan–Meier survival curve and log-rank test, Fig. 4c). In the MOA group (<i>n = 10</i> each), the complete metabolic panel (CMP) showed a drastic elevation of liver enzymes—both alanine aminotransferase (ALT) and alkaline phosphatase (ALP)—in fortin<sup>KO-liver</sup> mice compared with fortin<sup>WT-liver</sup> mice, suggesting that fortin<sup>KO-liver</sup> mice sustained far worse liver injury than did fortin<sup>WT-liver</sup> mice (Fig. 4d, ALT and ALP). Consistently, the liver function of fortin<sup>KO-liver</sup> mice was more severely compromised than that of fortin<sup>WT-liver</sup> mice upon EGF-SUBA challenge, as evidenced by higher total bilirubin levels (Fig. 4d, TBIL). There was no significant difference between EGF-SUBA-challenged fortin<sup>WT-liver</sup> and fortin<sup>KO-liver</sup> mice in hemoglobin/hematocrit (H/H) or white blood cell (WBC) counts (Supplementary Table 2). Upon gross inspection, the livers of fortin<sup>KO-liver</sup> mice were uniformly pale, indicating severe functional impairment of the organ (Fig. 4e, EGF-SUBA of fortin<sup>KO-liver</sup>). The kidney and pancreas were not affected by EGF-SUBA challenge (Supplementary Fig. 4D, blood urea nitrogen [BUN] and creatinine [CRE] for renal function and amylase [AMY] for pancreas). These data suggest that fortin<sup>KO-liver</sup> mice died of acute liver failure in response to EGF-SUBA challenge. Fortin blocks IRE1α from signaling apoptosis in the liver. We then tested whether fortin negatively regulated IRE1α in vivo, as we saw in the cellular and in vitro systems described above (Figs. 1, 2, and 3). Immunoblot analyses showed that fortin expression was severely decreased in the livers of fortin<sup>KO-liver</sup> mice regardless of treatment—PBS or EGF-SUBA (Fig. 4f, g, row a, lanes 1–4 vs. lanes 5–8; Supplementary Fig. 8). There was no difference between the livers of EGF-SUBA-challenged fortin<sup>KO-liver</sup> and fortin<sup>WT-liver</sup> mice in the levels of P-PERK, PERK, P-eIF2α, eIF2α, and ATF6F (Fig. 4f, IB and the bottom graph). In contrast, the phosphorylation of IRE1α (Fig. 4g, bands b2, b3, and b4 vs. bands b6, b7, and b8, and also the bottom graph) and JNK (Fig. 4g, bands d2, d3, and d4 vs. bands d6, d7, and d8, and also the bottom graph; and Supplementary Fig. 4E for ELISA of JNK and P-JNK) in the liver was more extensive in EGF-SUBA-treated fortin<sup>KO-liver</sup> mice than in EGF-SUBA-treated fortin<sup>WT-liver</sup> mice. The processing of XBP1 into the active form XBP1s by P-IRE1α was also greater in the livers of fortin<sup>KO-liver</sup> mice than in those of fortilin<sup>WT-liver</sup> mice (Fig. 4h, lanes 2–6 vs. lanes 8–12 for EGF-SUBA-treated fortin<sup>WT-liver</sup> and fortin<sup>KO-liver</sup> mice, respectively, also the bottom graph). Because XBP1s, a transcriptional factor, induces genes involved in ER-associated degradation (ERAD)<sup>3</sup>, we tested whether <i>Edem1</i> and <i>Herp1</i>—ERAD genes—are induced more robustly in the livers of fortin<sup>KO-liver</sup> mice than in those of fortin<sup>WT-liver</sup> mice in response to EGF-SUBA challenge. We performed quantitative reverse transcription polymerase chain reaction (RT-qPCR) on the total liver RNA and found that both <i>Edem1</i> and <i>Herp1</i> were induced more in the livers of EGF-SUBA-treated fortin<sup>KO-liver</sup> mice than in those of EGF-SUBA-treated fortin<sup>WT-liver</sup> mice (Fig. 4i). Hematoxyl and eosin (H&E) staining of SUBA-challenged liver sections also showed more drastic histopathological changes in fortin<sup>ko-liver</sup> mice than in fortilin<sup>WT-liver</sup> mice (Supplementary Fig. 4F, total injury score = 17 vs. 14, respectively). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining of the liver sections showed that the livers of fortilin<sup>KO-liver</sup> mice challenged by EGF-SUBA exhibited a higher degree of apoptosis than those of fortilin<sup>WT-liver</sup> mice (Fig. 4j, TUNEL; ***<i>P < 0.005</i> by two-tailed unpaired t-test). Consistent with the immunoblot analysis results (Fig. 4g, rows b and d), immunostaining showed higher levels of P-IRE1α and P-JNK in the livers of fortilin<sup>KO-liver</sup> mice than in those of fortilin<sup>WT-liver</sup> mice upon EGF-SUBA challenge (Fig. 4j, P-IRE1α, P-JNK; ***<i>P < 0.005</i> by two-tailed unpaired t-test). TUNEL positivity and the immunoreactivity of P-IRE1α and P-JNK in the PBS-treated livers of fortilin<sup>ko-liver</sup> and fortilin<sup>WT-liver</sup> mice were equally low (Supplementary Fig. 4G). These data suggest that fortilin in the liver protects whole animals against ER stress-induced liver damage and death by mitigating the activation of the IRE1α stress sensing pathway and hepatocyte apoptosis.
Because fortillin is an anti-apoptotic protein \(^{16, 20-22}\), however, we still do not know whether the apoptosis of fortillin-deficient hepatocytes is the result of (i) EGF-SubA-induced hyperactivation of the IRE1α pathway in the absence of fortillin or (ii) activation of other apoptotic pathways that are normally and negatively regulated by fortillin. To evaluate the exact role of fortillin in EGF-SubA-induced ER stress-mediated liver damage, we repeated the same experiment in the presence of the specific IRE1α inhibitor KIRA6 \(^{44}\) (Fig. 5a). KIRA6 (CAS Registry Number = 1589527-65-0, MW = 518.5) is an imidazopyrazine-based small-molecule IRE1α inhibitor that competitively binds the ATP binding site of the kinase domain (Fig. 3c) and blocks both the kinase and RNAse activities of the molecule \(^{46}\). We pretreated fortillin WT-liver and fortillin KO-liver mice with KIRA6 for 3 days, injected EGF-SubA (250 μg kg \(^{-1}\) body weight) once intraperitoneally on the third day, continued KIRA6 treatment for another 6 days, sacrificed the animals on the ninth day, and subjected the blood to CMP and complete blood count (CBC) and the livers to histological and molecular analyses. All animals survived for 9 days. Consistent with data shown in Fig. 4d, when treated with the vehicle (not KIRA6), fortillin KO-liver mice showed a drastically higher elevation of ALT than did fortillin WT-liver mice (Fig. 5b, Vehicle). When treated with KIRA6, however, neither the ALT (Fig. 5b, KIRA6) nor the ALP/TBL (Supplementary Fig. 5) levels of EGF-SubA-challenged fortillin KO-liver mice were statistically significantly different from those of fortillin WT-liver mice. There was no significant difference in H/H or WBC counts between EGF-SubA-challenged fortillin WT-liver and fortillin KO-liver mice, regardless of KIRA6 treatment (Supplementary Table 3). Upon gross inspection, the livers of fortillin KO-liver mice appeared similar to those of
fortilin WT-liver mice when treated with KIRA6 (Fig. 5c, KIRA6). Mechanistically, KIRA6 treatment did not change the way the PERK and ATF6 pathways responded to EGF-SubA in the presence and absence of fortilin in the liver (Fig. 5d; Supplementary Fig. 8). Although IRE1α was more phosphorylated in the livers of vehicle-treated fortilin KO-liver mice than in those of vehicle-treated fortilin WT-liver mice (Fig. 5e, bands b3 and b4 vs. bands b1 and b2, respectively; Supplementary Fig. 8), the degree of the phosphorylation of IRE1α in the livers of KIRA6-treated fortilin KO-liver mice was similar to and as low as that in the livers of KIRA6-treated fortilin WT-liver mice (Fig. 5e, bands b7 and b8 vs. b5 and b6, respectively). In addition, although JNK was more phosphorylated in the livers of vehicle-treated fortilin KO-liver mice than in those of vehicle-treated fortilin WT-liver mice (Fig. 5e, bands d3 and d4 vs. bands d1 and d2, respectively), the degree of the phosphorylation of JNK in the livers of KIRA6-treated fortilin KO-liver mice and was similar to that in the livers of KIRA6-treated fortilin WT-liver mice (Fig. 5e, bands d7 and d8 vs. d5 and d6, respectively). Further, when treated with KIRA6, the processing of XBP1 into the active form XBP1s was no longer significantly different between fortilin WT-liver mice and fortilin KO-liver mice (Fig. 5f, lanes 9–12 vs. lanes 13–16; also the right panel). Moreover, when treated with KIRA6, the livers of fortilin KO-liver mice challenged by EGF-SubA exhibited the same degree of lamin cleavage (i.e., apoptosis) as those of fortilin WT-liver mice (Fig. 5g). Finally, consistent with these immunoblot analysis results (Fig. 5e, rows b and d), immunostaining showed the same levels of P-IRE1α and P-JNK in the livers of fortilin KO-liver mice and fortilin WT-liver mice when the EGF-SubA-challenged mice were treated with KIRA6 (Fig. 5h, i). Taken together, these data suggest that fortilin blocked the majority of EGF-SubA-mediated liver damage—as evidenced by elevated ALT (Fig. 5b), ALP (Supplementary Fig. 5), and TBL (Supplementary Fig. 5) levels—and apoptosis (Fig. 5g) through its specific inhibition of the IRE1α pathway.

**Discussion**

The current work reveals yet another facet of the anti-apoptotic activity of fortilin. Previous studies have shown that fortilin exerts its anti-apoptotic activity through (a) its binding to and stabilization of MCL121, a BCL-2 family member and macrophage survival factor46, (b) binding to and de-stabilization of transforming growth factor-β-stimulated clone-22 (TSC-22), a pro-apoptotic protein47, (c) binding to Ca2+ and blockade of Ca2+–dependent apoptosis48, and (d) binding to and inhibition of the tumor suppressor protein p53s22. Based on the current work, we now know that fortilin also inhibits apoptosis by directly interacting with IRE1α in its cytosolic domain and blocking the activation of its downstream pathways—XBP1 and JNK (the latter leading to apoptosis). Importantly, fortilin selectively blocks the IRE1α pathway without affecting the PERK and ATF6 pathways (Figs. 2d–f, 4f–h, and 4j). Our current working model is as follows (Fig. 5j): Upon ER stress, IRE1α molecules, unphosphorylated and monomeric at the baseline4, form clusters with each other and start to trans-autophosphorylate each other4. As soon as IRE1α becomes phosphorylated (P-IRE1α), fortilin strongly binds to it (Fig. 3a, b, d, e, and Supplementary Fig. 3A) and prevents it from phosphorylating other IRE1α molecules (Fig. 2e, row b; Fig. 3f; Supplementary Fig. 3f; Fig. 4g, row b; Fig. 4j, P-IRE1α; Fig. 5e, row b). The fact that fortilin binds P-IRE1α (Fig. 3a, the right panel, lanes 3 and 4; Fig. 3e) and not IRE1α (Fig. 3a, the right panel, lanes 1 and 2; Fig. 3d) does not necessarily contradict the fact that fortilin decreases IRE1α phosphorylation, because P-IRE1α is the very kinase that phosphorylates IRE1α (trans-autophosphorylation). P-IRE1α, which is the activated form of IRE1α, activates both the JNK apoptosis pathway (Fig. 2e, row d) and the XBP1 pathway (Fig. 2e, row f; Fig. 2f) in a dose-dependent fashion. Importantly, our molecular docking data (Fig. 3i, j) suggest that the binding of fortilin to P-IRE1α prevents the molecule from gaining access to and phosphorylating the other IRE1α, as fortilin obliterates the kinase core of the activated P-IRE1α (Fig. 3i). The MD simulation data (Fig. 3j) support the notion that fortilin preferentially binds to P-IRE1α because the fortilin–P-IRE1α complex is more thermodynamically stable than the fortilin–IRE1α complex.

The UPR is designed to achieve two distinct biological goals: (i) the preservation of stressed cells through restoration of ER homeostasis and (ii) the elimination of afflicted cells through apoptosis when they are damaged beyond repair. No ER stress trigger is known to exert either homeostatic or apoptotic UPR alone49. Rather, ER stress activates all UPR signaling pathways and simultaneously produces both homeostatic and apoptotic outputs49. How does fortilin participate in a life-or-death decision of ER-stressed cells whose IRE1α pathway is activated? Because fortilin negatively regulates both JNK (pro-apoptotic) and XBP1 (ER homeostasis, pro-survival) pathways through its binding to and inhibition of P-IRE1α (Fig. 5j), the anti-apoptotic activity of fortilin could be overcome by its inability to mount sufficient pro-survival signals, leading to apoptotic death of the cells. Experimentally, however, fortilin consistently protected the cells (Fig. 1c–h, Supplementary Fig. 1D, E, Fig. 4j [TUNEL], and 5g) against apoptosis. A hint may lie in the fact that XBP1 mRNA splicing activity is only transiently activated in the early phase of the UPR49, 50, and that fortilin is gradually induced by various stresses—such as high concentration glucose, heat, and ROS—and remains upregulated for a longer duration than does XBP1 mRNA splicing activity. In the early phase of the UPR when fortilin exists at a lower concentration in the cell, the XBP1 pathway might promote cell survival by restoring its homeostasis. In the late phase of the UPR when the XBP1 mRNA splicing activity subsides49, 50, fortilin might sustain cell survival by blocking the JNK pro-apoptotic pathway. Further investigation is needed to understand how fortilin navigates the dichotomy between JNK-induced apoptosis and XBP1-mediated homeostasis and survival.

Although many proteins interact with IRE1α (Supplementary Fig. 3G), only three are known to bind IRE1α and negatively regulate the IRE1α pathway aside from fortilin. They are BAX inhibitor 1 (B1, also known as transmembrane Bax inhibitor motif containing 6 [TMBIM6])44, Jun activation domain-binding protein-1 (JAB1, also known as CSN5 and COPPS5)55, and receptor for activated C-kinase 1 (RACK1, also known as guanine nucleotide binding protein, beta polypeptide 2-like 1 [GNB2L1])56 (Supplementary Fig. 3G, h). B1 is a 295-aa transmembrane protein that binds to the cytosolic portion of IRE1α and inhibits the processing of XBP1 mRNA. JAB1, a 334-aa protein with a nuclear localization signal, binds to the linker region of the cytosolic portion of IRE1α in the absence of stress. Upon ER stress, JAB1 dissociates from IRE1α, allowing IRE1α to splice XBP1 mRNA into XBP1s, suggesting that JAB1 inhibits IRE1α through its binding55. It is unknown how the association between JAB1 and IRE1α is regulated or whether JAB1 prevents the kinase activity of IRE1α. RACK1, a 317-aa scaffold protein containing seven Trp-Asp 40 repeats, binds to the linker region of IRE1α56. RACK1 also binds to protein phosphatase 2A (PP2A), positions IRE1α and PP2A close to each other, and keeps IRE1α dephosphorylated and inactivated56. ER stress increases the association between RACK1 and IRE1α, and PP2A dissociates from RACK1 under ER stress, facilitating autophosphorylation and activation of IRE1α56. In the current work, we found that fortilin, a 172-aa protein with no homology to BI-1, JAB1, or RACK1, also binds to
the cytosolic portion of IRE1α. Unlike JAB1, which dissociates from IRE1α in response to ER stress, fortilin translocates from the nucleus to the cytosol upon ER stress, gains access to the cytosolic portion of IRE1α (Fig. 1a and Supplementary Fig. 1A), and preferentially binds to P-IRE1α (Fig. 3a, e, j). There, unlike BI-1, JAB1, or RACK1, fortilin uniquely inhibits both the kinase (Fig. 2e, row d; Figs. 3i and 4g, row d; Fig. 4j), P-JNK, Supplementary Figs. 2e and 4e) and RNase (Fig. 2e, row f; Figs. 2f, 3k, 4h, i) activities of IRE1α.

Rapidly growing tumors may outgrow their vascular supply and experience hypoxia and hypoglycemia.3 For tumor cells to adapt to and survive in this adverse microenvironment, activation of the UPR is crucial.12 In fact, the activation of IRE1α has been shown to facilitate angiogenesis and invasion of cancerous cells.58

Fortilin, an inhibitor of activated IRE1α (Fig. 5j) may inhibit IRE1α-mediated tumor growth and invasion in certain tumors. There has been no effort made to test whether fortilin is mutated and made dysfunctional in certain tumors. It is possible that certain fortilin mutations could make fortilin unable to bind IRE1α, thus activating the XBP1 homeostasis branch of the IRE1α pathway and facilitating tumor growth and propagation. In contrast, other tumors could take advantage of wild-type fortilin to block the activation of the IRE1α-JNK apotosis pathway to survive and propagate.

Hepatocytes, which produce and secrete a large amount of proteins, are naturally susceptible to ER stress. ER stress has been shown to contribute to various liver diseases.59 Results of the current study show that the lack of fortilin made the liver unusually susceptible to ER stress-induced dysfunction and injury (Figs. 4 and 5 and Supplementary Figs. 4 and 5). It is likely that fortilin would fortify the liver against ER stress-induced and IRE1α-mediated liver injury, which is known to occur in alcoholic liver disease60 and viral hepatitis61.

Further studies are needed to therapeutically exploit the newly unraveled physical and functional interaction between fortilin and IRE1α in human diseases. The fortilin[lox/lox] mice described herein (Supplementary Fig. 4A, B) should be a viable translational tool to evaluate the role of fortilin and the fortilin-IRE1α interaction in handling stress in a tissue-specific fashion.

Methods

Molecular cloning. For cloning of fortilin complimentory DNA (cDNA) into the pESG-IBA5-vector, the cDNA encoding human fortilin obtained previously in our laboratory16 was cloned into the multiple cloning site of the pESG-IBA5 mammalian expression vector (IBA Life Sciences, Göttingen, Germany) using a PCR-based strategy.

Cell culture and cell lines. Both U2OS (Catalog #: HTB-96) and PC3 (Catalog #: CRL-1435) cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). These cell lines were maintained in high-glucose Dulbecco’s modified Eagle’s medium and Roswell Park Memorial Institute (RPMI) 1640 medium, respectively, and supplemented with 10% fetal bovine serum (FBS). (HyClone, Logan, UT, USA) at 37 °C in an atmosphere containing 5% CO2. Both U2OS and PC3 were trypsinized using 0.05% trypsin-EDTA (Invitrogen, Carlsbad, CA, USA) every 2–3 days, and passaged with a ratio of 1:3–1:5. Cells were seeded in 12-well or 60-mm tissue-culture dishes (Thermo Fisher Scientific, Waltham, MA, USA) at a density of 1 × 105 per well. After 24 h, the cells were trypsinized and subjected to DNA fragmentation assay.

Immunoblot analysis. SDS-PAGE and immunoblot analyses were performed as described previously.34 The following primary antibodies were used at the indicated dilutions/concentrations: Mouse anti-ATF6 (dilution used = 1:250, Clone 70B1413.1, Imgenex-Novus Biologicals, Littleton, CO, USA); Mouse anti-α-tubulin (dilution used = 1:250, Clone 3B4, Santa Cruz Biotechnology, Santa Cruz, CA, USA); Mouse anti-β-actin (dilution used = 1:250, Clone C4, Santa Cruz Biotechnology); Goat anti-calnexin (dilution used = 1:250, sc-6465, Santa Cruz Biotechnology); Rabbit anti-calreticulin (dilution used = 1:1000, Clone D36E, #12328, Cell Signaling Technologies, Danvers, MA, USA); Rabbit anti-caspase-3 (dilution used = 1:1000, Clone 8G10, Cell Signaling Technologies), which was used to detect full-length p17, p19 caspase-3; Rabbit anti-caspase-9 (dilution used = 1:1000, #9502, Cell Signaling Technologies); Rabbit anti-phospho-mouse-eIF2α (dilution used = 1:1500, Se51, #9721, Cell Signaling Technologies); Rabbit anti-phospho-human-eIF2α (dilution used = 1:1500, Se51, #9721, Cell Signaling Technologies); Rabbit anti-phospho-mouse-eIF2α (dilution used = 1:1500, Se51, #9721, Cell Signaling Technologies); Mouse anti-fortilin (dilution used = 1:1000, Clone M305, Abnova, Taipei City, Taiwan), which was used for all experiments other than those shown in Supplementary Fig. 1A and Supplementary Fig. 1B; Rabbit anti-fortilin (dilution used = 1:250, PM017, MBL International, Woburn, MA, USA), which was used for the experiments depicted in Supplementary Fig. 1A and B; Rabbit anti-fortilin monoclonal antibody (concentration used = 14 μg/mL–1, Clone EPR5540, ab133568, Abcam), which was used in immunoprecipitation, as shown in Fig. 3b and Supplementary Fig. 3A; Mouse anti-GAPDH (dilution used = 1:1000, Clone 10R-G199a, Fitzgerald, Acton, MA, USA); Rabbit anti-GRP78 (dilution used = 1:1500, sc-13968, Santa Cruz Biotechnology); Mouse anti-HDAC2 (dilution used = 1:1000, Clone Y461, Abcam); Rabbit anti-HSP90 (dilution used = 1:1000, Clone D6F1, Cell Signaling Technologies); Rabbit anti-HSP90 (dilution used = 1:1000, #4872, Cell Signaling Technologies); Rabbit anti-IRE1α (dilution used = 1:500, sc-20790, Santa Cruz Biotechnology); Rabbit anti-phospho-IRE1α (dilution used = 1:1000, ab98187, Abcam); Rabbit anti-JNK (dilution used = 1:1500, sc-571, Santa Cruz Biotechnology); Rabbit anti-human-fortilin-JNK (dilution used = 1:500, #9933, Cell Signaling Technologies); Rabbit anti-phospho-mouse-JNK (dilution used = 1:1250, 07-175, EMD Millipore, Billerica, MA, USA); Rabbit anti-Alpha II S100 Lamin A (dilution used = 1:250, #2035, Cell Signaling Technologies); Rabbit anti-MBP (dilution used = 1:500, sc-13914, Santa Cruz Biotechnology); Mouse anti-NQO2 (dilution used = 1:250, Clone A-5, Santa Cruz Biotechnology); Rabbit anti-PARP (dilution used = 1:1000, #9542, Cell Signaling Technologies), which was used to detect full-length PARP; Rabbit anti-cleaved PARP (dilution used = 1:1000, Clone D6E10, #5625, Cell Signaling Technologies); Rabbit anti-human-PERK (dilution used = 1:500, #5683, Cell Signaling Technologies); Rabbit anti-mouse-PERK (dilution used = 1:500, sc-13073, Santa Cruz Biotechnology); Mouse anti-phospho-eIF2α (dilution used = 1:1000, Clone 4A4, EMD Millipore); Rabbit anti-fortilin (dilution used = 1:500, sc-7146, Santa Cruz Biotechnology). All antibodies were used with appropriate IRDye680LT- or IRDye800CW-conjugated secondary antibodies (LI-COR, Lincoln, NE, USA). The signal intensities of protein bands were quantified using the Odyssey Infrared Imaging System (LI-COR).

Subcellular fractionation. ER subcellular fractions were obtained from 1.5 × 108 PC3 cells treated with either 0.5 μM TG, 2 nM EGF-SubA, or dimethyl sulfoxide (DMSO) using the Subcellular Protein Fractionation Kit (Pierce, Rockford, IL, USA) according to the manufacturer’s instructions. The subcellular fraction of each fraction was determined using BCA methods (Bio-Rad, Hercules, CA, USA). Exactly 10 μg of total proteins were resolved by 12% SDS-PAGE and subjected to immunoblot analysis using anti-calreticulin (an ER marker35), anti-HSP90 (cytosol marker38), HDAC2 (a nuclear marker39), and anti-fortilin antibodies.

DNA fragmentation assay. The Cell Death Detection ELISA PLUS kit (Roche, Indianapolis, IN, USA) was used following the manufacturer’s instructions, with modifications described previously.22 For Fig. 1c, d, PC3-empty or PC3-empty (0.3 × 106 per well) were seeded into six-well plates. The next morning, cells were treated with various concentrations of TG (0, 0.5, and 1.0 μM) or EGF-SubA (0.2, 2, and 20 nM, SbTech, Brookfiled, CT, USA) for 24 h before they were harvested (both adherent and floating) and subjected to the same assay.42 For SubCytometric analysis (Fig. 1D, U2OS-empty and U2OS-empty (0.3 × 106 per well) were seeded into six-well plates. The next morning, cells were treated with DMSO or 0.5 μM TG for 24 h before they were harvested (both adherent and floating) and subjected to the same assay.42

Flow cytometric analysis of apoptotic and necrotic cells. To assess the relative contribution of apoptosis and necrosis to total cell death induced by TG and EGF-SubA, PC3 cells were stained with FITC-conjugated Annexin V and PI (1 μg/mL–1). Flow cytometry was performed using the manufacturers instructions (ThermoFisher-Invitrogen-Molecular Probes). The population separated into four groups: (i) live cells without apoptosis or necrosis (no red or green fluorescence), (ii) apoptotic cells without necrosis (positive green fluorescence and negative red fluorescence), (iii) necrotic cells without apoptosis (negative green fluorescence and positive red fluorescence), and (iv) cells exhibiting both necrosis and apoptosis (positive green and red fluorescence). Flow cytometry data are represented as mean ± s.d. of four independent experiments.
CTGGGTAGACCTCTGGGAG

Enzyme-linked immunosorbent assay (ELISA) was used to detect antibodies. For the cell-based assay, PC3sh-Fortilin and PC3sh-Empty cells were plated into 96-well plates (1 × 10⁴ cells per well), and incubated with rabbit anti-fortilin (MBL International) and mouse anti-PDI (Molecular Probes) antibodies. After washes, bound antibodies were detected with goat anti-rabbit secondary antibodies. Anti-fortilin (MBL International) and mouse anti-PDI antibodies were used to detect co-immunoprecipitated IRE1α, P-IRE1α, β-actin, and anti-α-tubulin antibodies were used to detect co-immunoprecipitated IRE1α, P-IRE1α, β-actin, and anti-α-tubulin antibodies. Because of the non-specific bindings occurred. Rabbit IgG was used as negative control to ensure that sufficient washing to eliminate non-specific bindings occurred. Rabbit IgG was used to rule out the non-specific interaction that withstood the wash conditions used in the protocol.

Generation of recombinant human fortilin. Affinity purification of recombinant human fortilin was performed using the Strep-tag purification system (IBA Life Sciences) as described previously. 293T cells stably expressing human fortilin tagged with the Strep-tag II (WSHPQFEK) at its N-terminal end were collected by trypsinization, washed in PBS, resuspended in buffer W (100 mM Tris HCl [pH 8], 150 mM NaCl, 1 mM EDTA), lysed by repeated freeze-thaw cycles, and sonicated to shear the genomic DNA. Cleared total cell lysate was then passed through a column packed with Strep-Tactin Superflow resin (IBA Life Sciences). The column was washed five times with Buffer W before the Strep-tagged fortilin was eluted with Buffer E (Buffer W plus 2.5 mM desthiobiotin). The fractions were pooled and concentrated using centrifugal filters (Amicon® EMD Millipore). The concentrated protein samples were buffer-exchanged into PBS using Zeba® Spin Desalting Columns (ThermoFisher Scientific). The final purification product was characterized by Coomassie and immunoblot analyses (Supplementary Fig. 3B, lane 9; Supplementary Fig. 3F, fortilin).

Dephosphorylation of P-IRE1α. Dephosphorylation of recombinant P-IRE1α was performed using calf intestinal alkaline phosphatase (CIP)–agarose beads according to the manufacturer’s instructions (Sigma-Aldrich). Recombinant human phospho-IRE1α (0.4 µg protein per sample, Origene, Rockville, MD, USA) was resuspended in reaction buffer (50 mM Tris HCl, 10 mM NaCl, 1 mM MgCl₂, 0.1 mM dithiothreitol (DTT), pH 7.9) and treated with CIP–agarose (1 unit of CIP per 0.4 µg of protein) at 37 °C for 2 h. The reaction was stopped by separating the bead-bound enzyme from the reaction mixture by centrifugation at 9,000 × g. The CIP-treated sample was subjected to immunoblotting using rabbit anti-phospho IRE1α antibody (Abcam) (Supplementary Fig. 3E).

Bio-layer interferometry. Recombinant human fortilin protein produced as described above was biotinylated and immobilized on streptavidin-coated biosensors (Fortebio, Menlo Park, CA, USA) at a concentration of 25 pM. For 60 s, followed by buffer exchange in PBS. The system was tested using anti-fortilin antibody (positive control, Abnova) and bovine serum albumin (negative control, Sigma-Aldrich) as described in Supplementary Fig. 3C. D. Next, various concentrations of recombinant human phospho-IRE1α–Myc-DDK (aa 1–977, Origene, de-phosphorylated IRE1α–Myc-DDK (aa 1–977, Origene, see above for the dephosphorylation procedure), or anti-α-tubulin and anti-α-actin antibodies were used to detect co-immunoprecipitated IRE1α, P-IRE1α, β-actin, and anti-α-tubulin antibodies. Because of the non-specific bindings occurred. Rabbit IgG was used as negative control to ensure that sufficient washing to eliminate non-specific bindings occurred. Rabbit IgG was used to rule out the non-specific interaction that withstood the wash conditions used in the protocol.

Molecular docking assay of fortilin and IRE1α. To study how fortilin and IRE1α interact with each other, we performed a molecular docking experiment using Piper software on the ClusPro Server 2.0 and the crystal structures of fortilin (2HR9) and IRE1α (4U6R) available from the Protein Data Bank. We evaluated
rocking results using clustering analysis and weighted model scores as described by Kozakov et al.\textsuperscript{71} From the top 10 cluster populations, we selected the binding pose that best approximated the experimental state and used it for the determination of binding free-energy by MD simulations. We used the Gromacs v5.0.4 software package utilizing the GROMOS 54A7 force-field parameter set\textsuperscript{43}. We adopted force-field parameters for post-translational modifications of phosphorylation from Hansson et al.\textsuperscript{73} and applied them to the GROMOS 54A7 force field as previously described\textsuperscript{75}. An initial 100 ns MD simulation of fortilin bound with IRE1\textalpha was performed using the binding configuration obtained from the protein-protein docking studies. Simulations were performed using periodic boundary conditions with explicit solvation using a simple point charge model poten- tial. To prepare the system for simulation, a non-bonded cutoff of 10 Å was used, minimizing non-bonded interactions with a power dependency set to 1. At each step, the positions of all atoms were updated using the Verlet algorithm\textsuperscript{74}. The Verlet cutoff scheme was used in calculation of short-range Coulomb steps. All bonds were constrained to equilibrium values using the LINCS algo-

In vitro IRE1\textalpha Ntase activity assay. The endoribonuclease (RNase) activity of IRE1\textalpha in the presence of fortilin was assayed in vitro using fluorescence resonance energy transfer (FRET)-based methods described previously\textsuperscript{39}. Briefly, recombinant human IRE1\textalpha (10 μg, SignalChem) was pre-incubated with various concentrations of recombinant human fortilin (0–4 μg) in Kinase Buffer (20 mM HEPES, pH 7.5, 5 mM MgCl\textsubscript{2}, 1 mM EGTA, 0.02% Brij35, 0.02 mg mL\textsuperscript{-1} BSA, 0.1 mM Na\textsubscript{2}VO\textsubscript{4}, 2 mM DTT, and 1% DMSO) and incubated for 20 min at room temperature. The reaction was initiated by addition of [γ-33P]ATP (10 μM), carried out for 2 h at room temperature, and terminated by spotting the reaction mixture onto a phospho-

Generation of liver-specific fortilin knockout mice. Fortilin\textsuperscript{lox/lox} mice—in which the fortilin gene was flankned by the LoxP sequence to allow tissue-specific deletion—were generated using the standard homologous recombination technique as previously described\textsuperscript{37}. First, a mouse BAC clone containing the full fortilin gene was isolated from the C57BL/6 ES BAC clone library. A fortilin targeting vector was constructed by subcloning (a) the 4.8 kilobase (kb) upstream genomic sequence, (b) the 3.6 kb genomic DNA sequence containing all six fortilin exons (exon 1–exon 6), and (c) the 3.2 kb downstream genomic DNA sequence into the pBPVRTCRK targeting vector (Supplementary Fig. 4A, #2) that already contained (a) two LoxP sequences, (b) a phosphoglycerate kinase promoter-driven neomycin resistance gene flankned by two flippase recognition target sequences, and (c) the thymidine kinase cassette. After extensive sequencing to verify the integrity of the construct, the targeting vector was linearized by 1-CEu (R0699S, New England BioLabs, Ipswich, MA, USA) and electroporated into C57BL6 embryonic stem cells (ESCs). ESC clones that survived in neomycin-containing media were analyzed by PCR and Southern blotting (using radioactive DNA probes 1 and 2 after NdeI R0111S, New England BioLabs and NsiI R0127S digestion, respectively) for successful homologous recombination (Supplementary Fig. 4B). Two mutated ESC lines (B43 and B61) were microinjected into C57BL6 blastocysts, which were then incubated in pseudopregnant (pseudopregnant) mice to obtain chimera. The chimera were then crossed with C57BL6 mates, and their offspring were tested using PCR-based methods for germline transmission. The results for fortilin\textsuperscript{lox/lox} mice were fully in the C57BL6/6J genetic background from the beginning. Fortilin\textsuperscript{lox/lox} mice were then crossed with a transgenic flippase strain to remove the neomycin resistance gene cassette. To generate a new generation of liver-specific deletion, fortilin\textsuperscript{lox/lox} mice were crossed with C57BL/6J mice overexpressing the Cre recombinase transgene under the control of albumin enhancer/promoter (Stock #: 0003574, Jackson Laboratory, Bar Harbor, ME, USA) to generate liver-specific deletion of fortilin. Because the expression levels of fortilin in the livers of the Alb-

In vitro IRE1\textalpha kinase activity assay. The kinase activity of IRE1\textalpha in the presence of fortilin was assayed in vitro using a [γ-33P]ATP or [γ-32P]pATP to label a phosphorylation substrate (MBP, SignalChem) as a universal kinase substrate\textsuperscript{76} to the reaction mixture. The reaction mixture was incubated at 30°C for 45 min and subjected to SDS-PAGE and immunoblot analysis using anti-phosphoserine (EMD Millipore), anti-MBP (Santa Cruz), anti-fortilin (Abnova), and anti-phospho-IRE1\textalpha (Abcam) antibodies. The phosphorylation index was calculated by dividing the signal intensity of phosphorylated MBP by that of total MBP; results are expressed as A.U.\textsuperscript{2} Two independent experiments were performed with consistent findings (Supplementary Fig. 3F).

Mouse model of ER stress-induced liver damage. For EGF-SubA treatment, 5-week-old male fortilin\textsuperscript{K0}/\textsuperscript{L0} and fortilin\textsuperscript{WT}/\textsuperscript{WT} mice were intraperitoneally injected with 5 nmol EGF-SubA (40 nmol EGF+50 nmol SubA) for 3 weeks.
administered either PBS or EGF-SubA (Sibtech). For survival analyses, mice were kept on a 12 h dark-light cycle and had access to food and water ad libitum. They were examined daily for their behavior and health. For the mechanism of action (MOA) analyses, mice were sacrificed by CO2 inhalation and cervical dislocation 7 days after injection, and whole blood and organs were collected for further analyses. There was no blinding performed. For Kira6 treatment, 5-week-old male fortinlNKO-liver and fortilinKO mice were intraperitoneally administered either 5 mg kg−1 Kira6 (Catalog #: 532281, EMB Millipore) in solution made of 3% ethanol, 7% Tween-80, 90% saline or the same solution without Kira6 as vehicle daily for 9 days. On day 3 of the experiment, mice were intraperitoneally injected with 250 μg kg−1 of EGF-SubA (SibTech) once. Mice were examined daily for their behavioral health. A total of 9 days before treatment, sera and blood and organs were collected for further analyses (n = 4 each for immunoblot and XBP splicing assays, n = 6 for CRC, CMP, and immunohistochemistry) as described below. There was no blinding performed.

Whole-blood analyses. For the CMP and CRC, whole blood was sampled by cardiocentesis using a 25-gauge needle immediately after euthanasia by CO2 and cervical dislocation. The sample was transferred into Microtainer® tubes with lithium heparin (BD Biosciences, Franklin Lakes, NJ, USA) by removing the needle from the syringe, pouring the blood into the tube, and mixing it thoroughly with the lithium heparin. The blood chemistry profile was obtained using the Comprehensive Diagnostic Panel rotors and the Vetscan VS2 (Abaxis, Union City, CA, USA). The following blood chemistry parameters were assayed for each sample: albumin (ALB), AFP, ALT, AMY, TBIL, BUN, total calcium (Ca2+), phosphorus (PHOS), CRE, glucose (GLU), sodium (Na+), potassium (K+), total protein (TP), and globulin (GLOB). The results are shown in Figs. 4d and 5b and Supplementary Figs. 4D and 5. CRC was obtained using the HEMAVET 950FS Hematology System (Drew Scientific, Dallas, TX, USA). The CRC results are shown in Supplementary Tables 2 and 3.

Quantitative reverse transcription polymerase chain reaction. RT-qPCR was performed as described previously.22 Briefly, the lysates of fortilinWT-liver and fortilinKO-liver mice were harvested into Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA). RNA was isolated in accordance with the manufacturer’s instructions and treated with DNase (ABI, Foster City, CA, USA). RT-qPCR was performed in quadruplicate with exactly 50 ng of total RNA using the TaqMan® RT-qPCR kit (Applied Biosystems [ABI] at Life Technologies, Grant Island, NY, USA) in the ABI Step One Plus Real-Time PCR system and the following primer and probe sets (Integrated DNA Technologies): Mouse EDEM1-forward: 5′-TCT GGTGATGCTTGATGATC3′, reverse: 5′-GACCTGGACTGTGAAATCTT-3′, probe 5′-FAM-CCGAGTTCZEN/AGAAGGCGTAAGTTC-IAbkFQ-3′ where FAM = carboxyfluorescein, IAbkFQ = Iowa Black FQ, and ZEN = an internal quencher to enhance the quenching activity of the 3′ quencher Iowa Black FQ; Mouse Herp1-forward: 5′-CAACCTGGACATCTTGGGAC-3′, reverse: 5′-CAGATCGCCGACAGGAAATC-3′, probe 5′-FAM-CCGAGTTCZEN/AGAAGGCAGTAAGTT-IAbkFQ-3′, where FAM = carboxyfluorescein, IAbkFQ = Iowa Black FQ, and ZEN = a fluorescent quencher (Iowa Black FQ); and Mouse 18S ribosomal RNA (rRNA) Forward: 5′-CCAACCGAGAAGACAGAAGCCAGACAAAAGG-IAkbFQ and Mouse 18S ribosomal RNA (rRNA)-forward: 5′-GCCGCTAGAGGTGATATCTT-3′, reverse: 5′-TGAGAGACTGAGGCTTCT-3′, probe: 5′-JOEN-ACCAGAGGCGZEN/AAACGTGGCAAGCC1AbkFQ-3′ where JOEN = 6-carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein.

Hematoxylin and eosin staining. The formalin-fixed H&E sections of liver tissue were evaluated on day 7 post-SubA challenge for histopathological changes. The various parameters assessed included steatosis (macro- and micro-vesicular), apoptosis, mitosis, inflammation, pattern of injury (for example, zones 1, 2, or 3), and markers of injury such as the presence of giant mitochondria or Mallory’s hyaline. The sections (n = 6 each for SubA-treated fortilinWT-liver and fortilinKO-liver mice; n = 3 for PBS-treated fortilinKO-liver mice; n = 2 for PBS-treated fortilinWT-liver mice) were blindly evaluated by an experienced hepatopathologist, who graded the above features on a scale of 0-4. Apoptotic and mitotic counts were assessed semi-quantitatively by counting the number per a field. The numbers of mice used in in vivo experiments were determined by (i) power analysis, assuming a error rate of 0.05, β error rate of 0.20, and expected difference of 25% and using Minitab 17 (State College, PA, USA) or (ii) our previous data set and experience from the similar experiments performed in the past.

Data availability. The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. All relevant data are available from the authors upon request.

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**Author contributions**

K.F. conceived the general idea and framework of the project, designed the majority of the experiments, and oversaw the project to its completion. D.P. contributed to the experimental design and performed most of the experiments, including in vitro, cellular, and animal experiments. D.P. and K.F. analyzed data and composed figures. A.C. performed the PLA experiments. P.C. performed the flow cytometric analysis of apoptotic and necrotic cells. P.S. performed the XBP splicing assay of mouse livers. Z.L. performed subcellular fractionation and the cell-based survival and apoptotic assay using thapsigargin. K.F. directed the generation and characterization of fortilinlox/lox mice. Y.C. and K.F. generated fortilinlox/lox mice. D.P. and K.F. generated fortilinKO-liver mice. H.L.S. graded liver H&E sections in a blinded fashion. M.D.K. and O.M.M. performed the docking and molecular dynamics simulation experiments. D.P., M.D.K. and K.F. wrote the manuscript. K.F., D.P., O.M.M. and A.C. proofread the manuscript.

**Additional information**

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