Supporting Information

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SI Materials and Methods

Retrovirus Production and Cell Transduction. N-terminally Flag-M1–tagged human IRE1α (19–977) [full-length (FL)-IRE1α] was produced as previously described (1). cDNA encoding N-terminally Flag-M1–tagged human IRE1α deleted of the luminal residues P_{29}–D_{408} (ΔLD-Ire1α) and N-terminally Flag-M1–tagged human IRE1α Y_{464}–A_{977} corresponding to the cytosolic domain (cyto-IRE1α) were derived from the full-length protein by PCR. We mutated the transmembrane domain of FL-IRE1α and ΔLD-IRE1α by exchanging the wild-type transmembrane domain (TM-WT) $_{442}$*KD*MATIILSTFLLIGWVAFIITY*PLSMH*$_{469}$, to a transmembrane domain with a scrambled amino acid sequence (TM-SC) *KD*YVLTMFISITWIAIFTGLLIA*PLSMH* or to the transmembrane domain of calnexin (TM-CNX) *KD*EERPWLWV-VYILTVALPVFLVILFCCSGKK*MH*. The amino acids in italics are outside of the predicted transmembrane of IRE1α. The underlined amino acids are present in the predicted transmembrane domains.

A Flag-M1–tagged PERK deleted of the luminal domain (ΔLD-PERK) was constructed by fusing the preprotrypsinogen signal peptide and the Flag-M1 tag to a C-terminal fragment containing the transmembrane and cytoplasmic effector domains of mouse PERK (S_{503}–N_{1113}). The corresponding cDNAs were introduced into the pBABE-puromycin retroviral vector. All plasmids were sequenced to verify that they correspond to the expected sequence. Retroviral particles produced in 293T cells were used to infect IRE1α$^{-/-}$ (2) or PERK$^{-/-}$ (3) cells. Forty-eight hours after infection, the cells were placed in selection medium containing puromycin (3 μg/mL). We picked and expanded single colonies of cells and verified the expression of the transgene by Western blot. Cells were maintained in selection medium.

Immunoprecipitation and Immunoblotting. Cells were lysed as previously described (4). Anti-Flag affinity gel (Sigma) was used to immunoprecipitate Flag-tagged IRE1α and PERK followed by immunoblotting with rabbit serum to IRE1α or PERK. IRE1α phosphorylation was analyzed by SDS/PAGE in the presence of 50 μM Phos-tag reagent (NARD Institute) and 100 μM $MnCl_2$ (5). Antisera to IRE1α, PERK, ATF4, and eIF2α were raised in rabbit and used as previously described (4, 6). Anti-Flag antibodies (Sigma), anticalnexin (Abcam), phospho-EGF receptor (D7A5), and anti-EGF receptor (D38B1) (Cell Signaling Technology) were obtained commercially and used according to the manufacturer's instruction.

***Xbp1* mRNA Splicing Assay and Quantitative PCR.** RNA was extracted using RNeasy columns (Qiagen). *Xbp1* mRNA splicing was determined by RT-PCR (2) using the One-Step RT-PCR kit (Qiagen), the amplified product stained with SYTO-60 (Invitrogen), and visualized on an Odyssey scanner (Li-Cor). Quantitative PCR analysis was performed using the SYBR Green reagent system (Applied Biosystems) and an ABI 7900 (PerkinElmer). Relative quantities of amplified cDNAs were then determined using SDS software (Applied Biosystems).

Bacterial Protein Expression. A bacterial expression plasmid 6×-His-ΔLD-PERK-pQE30 encoding an N-terminally 6 histidine-tagged fragment of mouse PERK lacking the luminal domain, but containing the transmembrane domain and the cytosolic kinase domain (amino acids S_{503}–N_{1114}), was used to transform M15 bacteria. A bacterial expression plasmid 6×-His-PERK-KD-pET30a encoding an N-terminally 6 histidine-tagged fragment of mouse PERK lacking the luminal domain and the transmembrane domain, but containing the cytosolic kinase domain (amino acids R_{537}–N_{1114}), was used to transform BL21 bacteria. Following induction with 1 mM isopropylthio-β-galactoside at OD_{600} = 0.6, bacteria were cultured for 16 h at 20 °C. Bacteria were lysed in buffer containing 1% Triton X-100 by processing the cells through a high-pressure microfluidizer (Avestin; EmulsiFlex-C3). Lysates were clarified by ultracentrifugation, purified using a Mini His Trap column (GE Healthcare), and eluted with 300 mM imidazole.

Kinase Assays. Proteoliposomes were diluted on ice in kinase buffer and incubated at 37 °C in the presence of 10 μM of [γ-^{32}P] ATP (corresponding to 8 μCi). Reactions were quenched by addition of 2× SDS dye, products were separated by SDS/PAGE and stained with Coomassie Instant Blue (Expedion), and the dried gel was subjected to autoradiography. Coomassie stain was visualized on an Odyssey scanner (Li-Cor). Phosphorylation of eIF2α was carried with diluted proteoliposomes in kinase buffer containing 20 μM of bacterially expressed mouse eIF2α N-terminal fragment (1–185) and 0.2 mM cold ATP at 37 °C. Reactions were quenched by addition of 2× SDS dye, products were separated by SDS/PAGE containing 50 μM Phos-tag reagent (NARD Institute) and 100 μM $MnCl_2$, stained with Coomassie Instant Blue (Expedion). Coomassie stain was visualized on an Odyssey scanner (Li-Cor).

1. Cross BC, et al. (2012) The molecular basis for selective inhibition of unconventional mRNA splicing by an IRE1-binding small molecule. *Proc Natl Acad Sci USA* 109(15): E869–E878.
2. Calfon M, et al. (2002) IRE1 couples endoplasmic reticulum load to secretory capacity by processing the *XBP-1* mRNA. *Nature* 415(6867):92–96.
3. Harding HP, Zhang Y, Bertolotti A, Zeng H, Ron D (2000) *Perk* is essential for translational regulation and cell survival during the unfolded protein response. *Mol Cell* 5(5):897–904.
4. Bertolotti A, Zhang Y, Hendershot LM, Harding HP, Ron D (2000) Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nat Cell Biol* 2(6): 326–332.
5. Yang L, et al. (2010) A Phos-tag-based approach reveals the extent of physiological endoplasmic reticulum stress. *PLoS ONE* 5(7):e11621.
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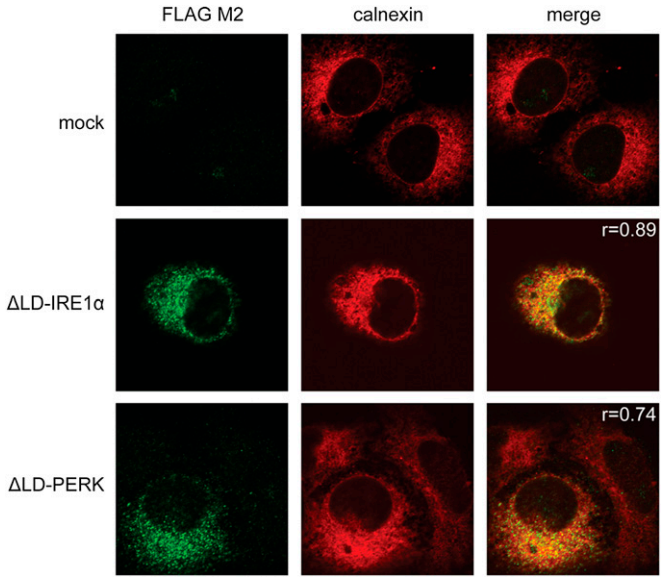

Fig. S1. Intracellular localization of ΔLD-IRE1α and ΔLD-PERK. Photomicrographs of COS7 cells transfected with empty pBABE-puromycin (mock) or pBABE-puromycin expressing Flag-tagged ΔLD-IRE1α or Flag-tagged ΔLD-PERK and stained, 24 h posttransfection, with mouse-anti-Flag M2 and rabbit-anticalnexin and the secondary antibodies goat anti-mouse Dye Light 488 and goat anti-rabbit Dye Light 543. Images were taken on a Zeiss Meta 510 confocal microscope using sequential acquisition. Colocalization of ΔLD-IRE1α and ΔLD-PERK with the ER marker calnexin was quantified by Pearson's coefficient (*r*).

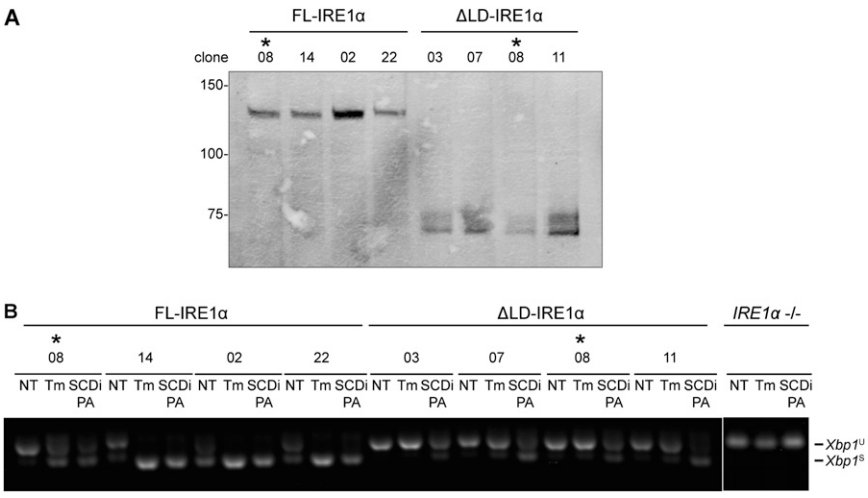

Fig. S2. Analysis of IRE1α-dependent XBP1 mRNA splicing in clones of *IRE1α*$^{-/-}$ cells transduced with FL-IRE1α or ΔLD-IRE1α. (*A*) Immunoblot of IRE1α immunopurified from individual clones of retrovirally transduced *IRE1α*$^{-/-}$ cells. Note the comparable expression of the FL-IRE1α and ΔLD-IRE1α in the clones chosen for the experiments presented in the main text (marked by the asterisks). (*B*) RT-PCR analysis of *Xbp1* mRNA purified from the clones shown above and the parental *IRE1α*$^{-/-}$ cells. Cells were exposed for 4 h to tunicamycin (Tm; 5 μg/mL) or were treated for 24 h with the SCD1 inhibitor (SCD1i) CVT-11127 at 1 μM, followed, when indicated, by exposure to SCD1i in the presence of 0.5 mM palmitic acid (PA). The position of the unspliced (*Xbp1*^{U}) and spliced (*Xbp1*^{S}) product is indicated. Note, that despite variation in baseline signal among the clones, *Xbp1* mRNA splicing is not increased by tunicamycin but is increased by lipid saturation in all four ΔLD-IRE1α transduced lines.

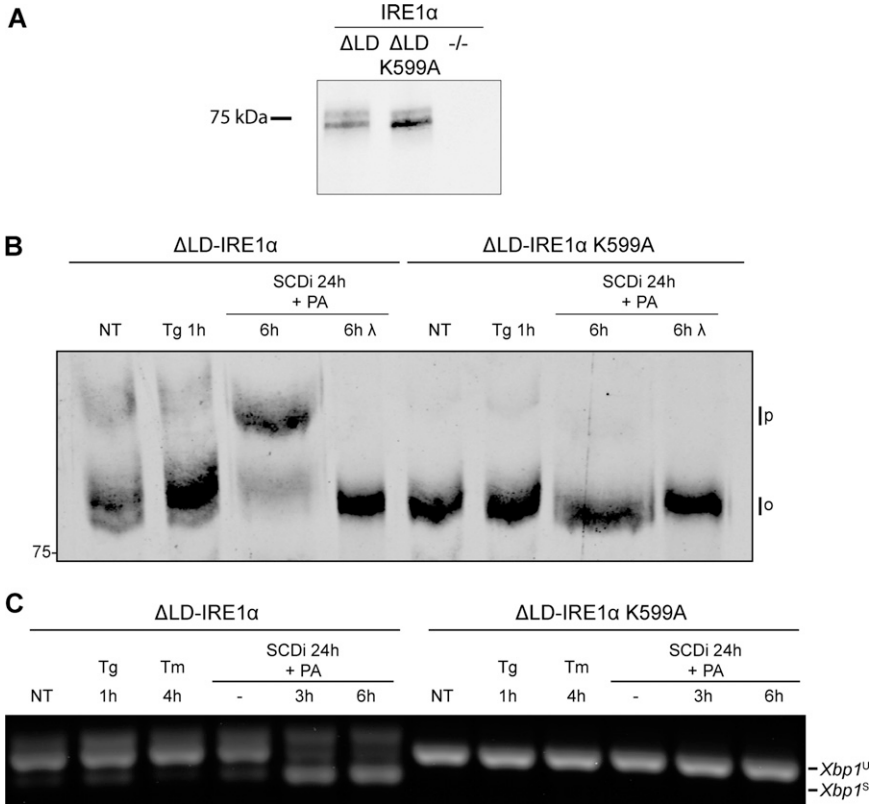

Fig. S3. Autokinase functionality is required for lipid perturbation-mediated *Xbp1* mRNA splicing by ΔLD-IRE1. (*A*) Flag-M2 immunoblot of wild type and kinase domain mutant (K599A) ΔLD-IRE1α immunopurified from transduced *IRE1α*$^{-/-}$ cells. (*B*) Immunoblot of a Phos-tag gel of the wild type and K599A mutant ΔLD-IRE1α immunopurified from transduced *IRE1α*$^{-/-}$ cells that had been exposed to thapsigargin (Tg) or an SCD1 inhibitor and palmitic acid. λ-Phosphatase was applied in vitro to the purified proteins, where indicated. (*C*) RT-PCR analysis of *Xbp1* mRNA purified cells treated as above.

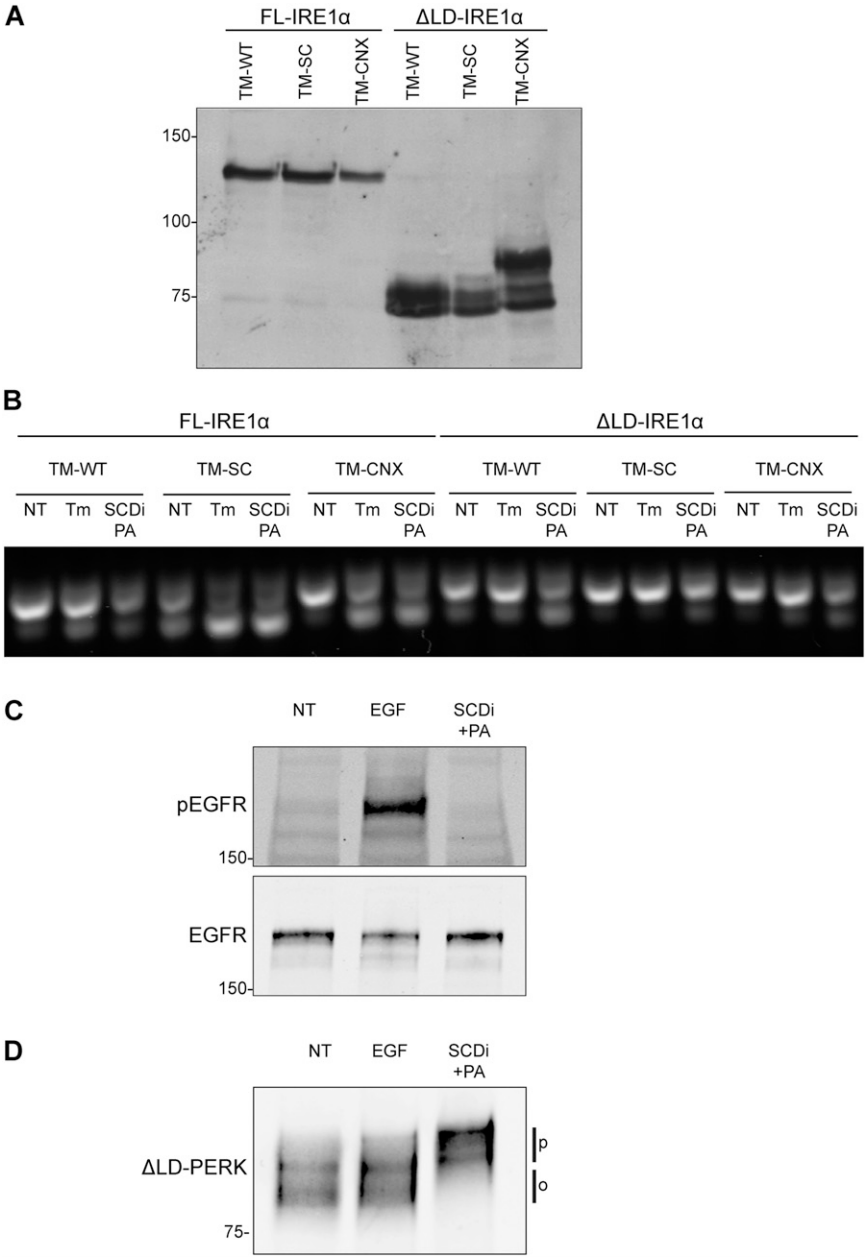

Fig. S4. Relaxed specificity of transmembrane domain amino acid sequence for IRE1α activation by lipid perturbation. (*A*) Immunoblot of IRE1α derivatives immunopurified from reconstituted *IRE1α*$^{-/-}$ cells. FL-IRE1α and ΔLD-IRE1α were constructed with their wild-type transmembrane domain (TM-WT) a sequence-scrambled transmembrane domain (TM-SC), or the transmembrane domain of calnexin (TM-CNX). The anomalous migration of ΔLD-IRE1α TM-CNX was a reproducible feature of several clones. (*B*) RT-PCR analysis of *Xbp1* mRNA of the cells shown in *A*. Cells were exposed for 4 h to tunicamycin (Tm; 5 μg/mL) or treated for 24 h with the SCD1 inhibitor (SCDi) CVT-11127 at 1 μM, followed, when indicated, by exposure to SCDi in the presence of 0.5 mM palmitic acid (PA). The position of the unspliced (*Xbp1*^{U}) and spliced (*Xbp1*^{S}) product is indicated. Note, that despite variation in activity, substitution of the transmembrane domain did not abolish the response to lipids. (*C*) Immunoblot of activated, phosphorylated, endogenous epidermal growth factor receptor (pEGFR) and total EGFR from HEK293T cells exposed to EGF (150 ng/mL, 20 min) or SCDi in the presence of 0.5 mM palmitic acid (PA). (*D*) Immunoblot of ΔLD-PERK immunopurified from the transfected HEK293T cells shown in *C*.

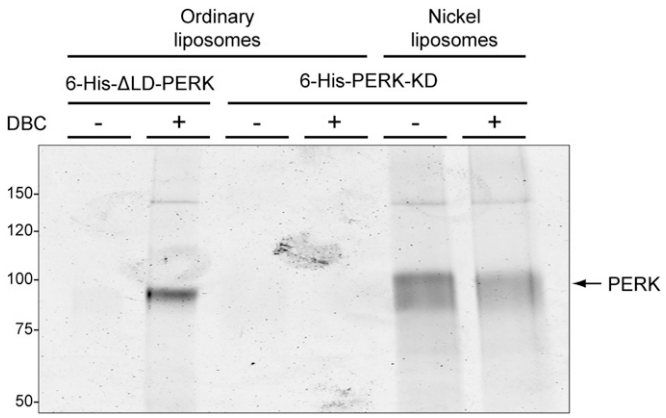

Fig. S5. Determinants of PERK proteoliposomes formation. Coomassie-stained SDS/PAGE of 6-His-ΔLD-PERK and 6-His-PERK-KD proteoliposomes recovered after flotation. Ordinary liposomes or nickel-NTA–bearing liposomes were incubated with the indicated proteins in the presence or absence of 0.25% of the detergent deoxy-Big CHAP (DBC) followed by Biobead SM2 extraction of the detergent and flotation.