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Supplemental Information

The Unfolded Protein Response Element IRE1α Senses Bacterial Proteins Invading the ER to Activate RIG-I and Innate Immune Signaling

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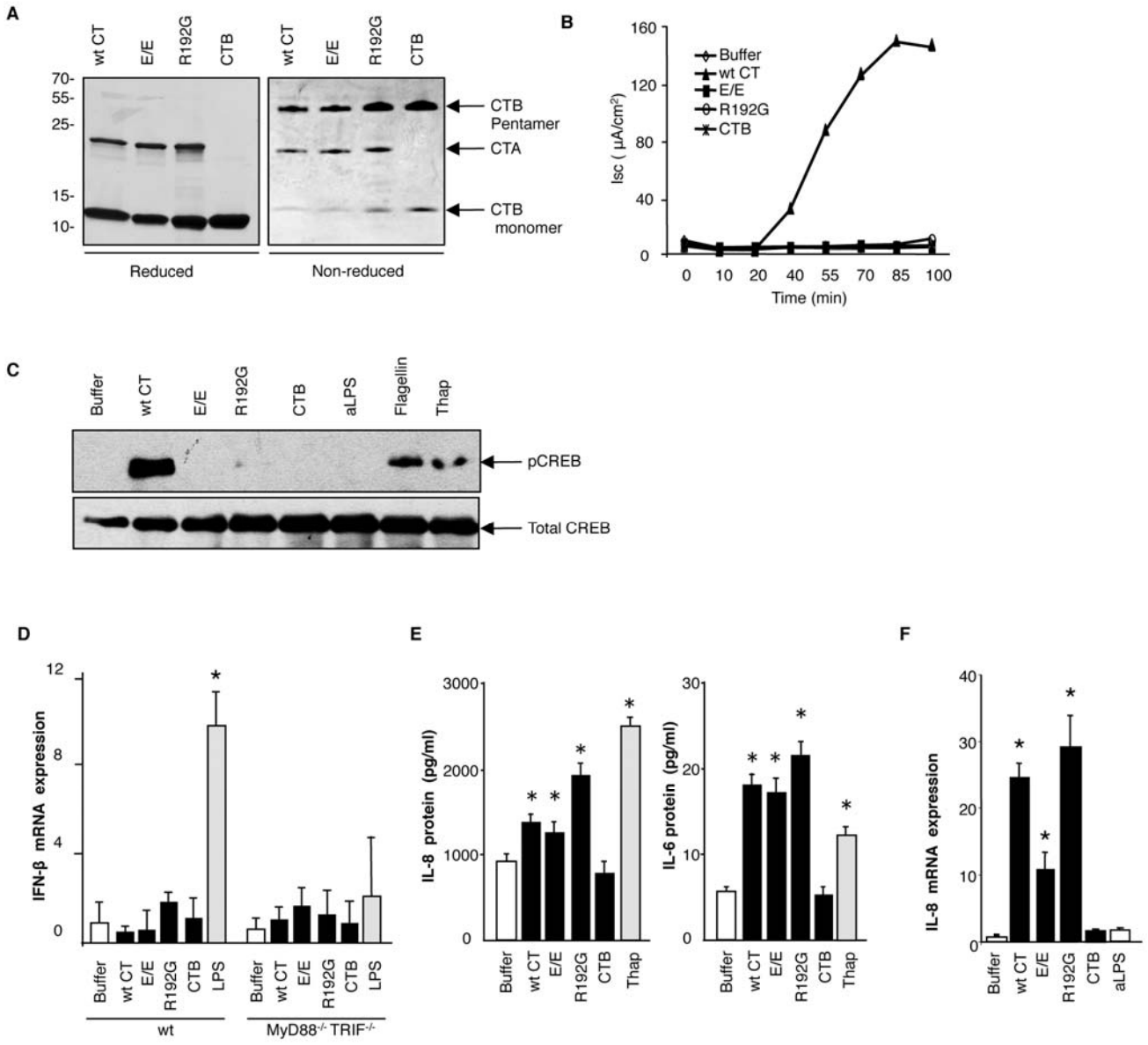

Figure S1. The E/E and R192G mutants and CTB are not toxic and do not contain detectable levels of LPS, Related to Figure 1

(A) Coomassie stain of the toxins used in this study. Three μg of toxin was loaded onto the SDS-PAGE in the presence or absence of β-mercaptoethanol.

(B) Toxin-induced cAMP-dependent Cl^- secretion measured as a short-circuit current (Isc) in T84 cells intoxicated apically with 3 nM toxins.

(C) Immunoblot for phosphorylated cAMP-response element binding (CREB) for toxin-induced activation of adenylyl cyclase.

(D) Q-PCR analysis of IFN-β mRNA expression in primary bone marrow-derived macrophages from wild-type (wt) and MyD88/TRIF double knockout ($MyD88^{-/-}$ $TRIF^{-/-}$) mice treated with 3 nM toxins for 4h. $MyD88^{-/-}TRIF^{-/-}$ confirmed specificity for TLR4 signal transduction.

(E) Cytometric bead array analysis of IL-8 and IL-6.

(F) Q-PCR analysis of IL-8 mRNA expression in T84 cells intoxicated with 40 nM toxins for 4h.

Open bars represent negative controls. Gray bars represent positive controls. LPS apically (aLPS) as negative controls, flagellin or thapsigargin (Thap) basolaterally as positive controls. Total CREB as loading control.

wt CT, wild type CT; E/E, E110D/E112D mutant; R192G, R192G mutant; CTB, B-subunit alone. Data are means ± SEM. *$p<0.05$

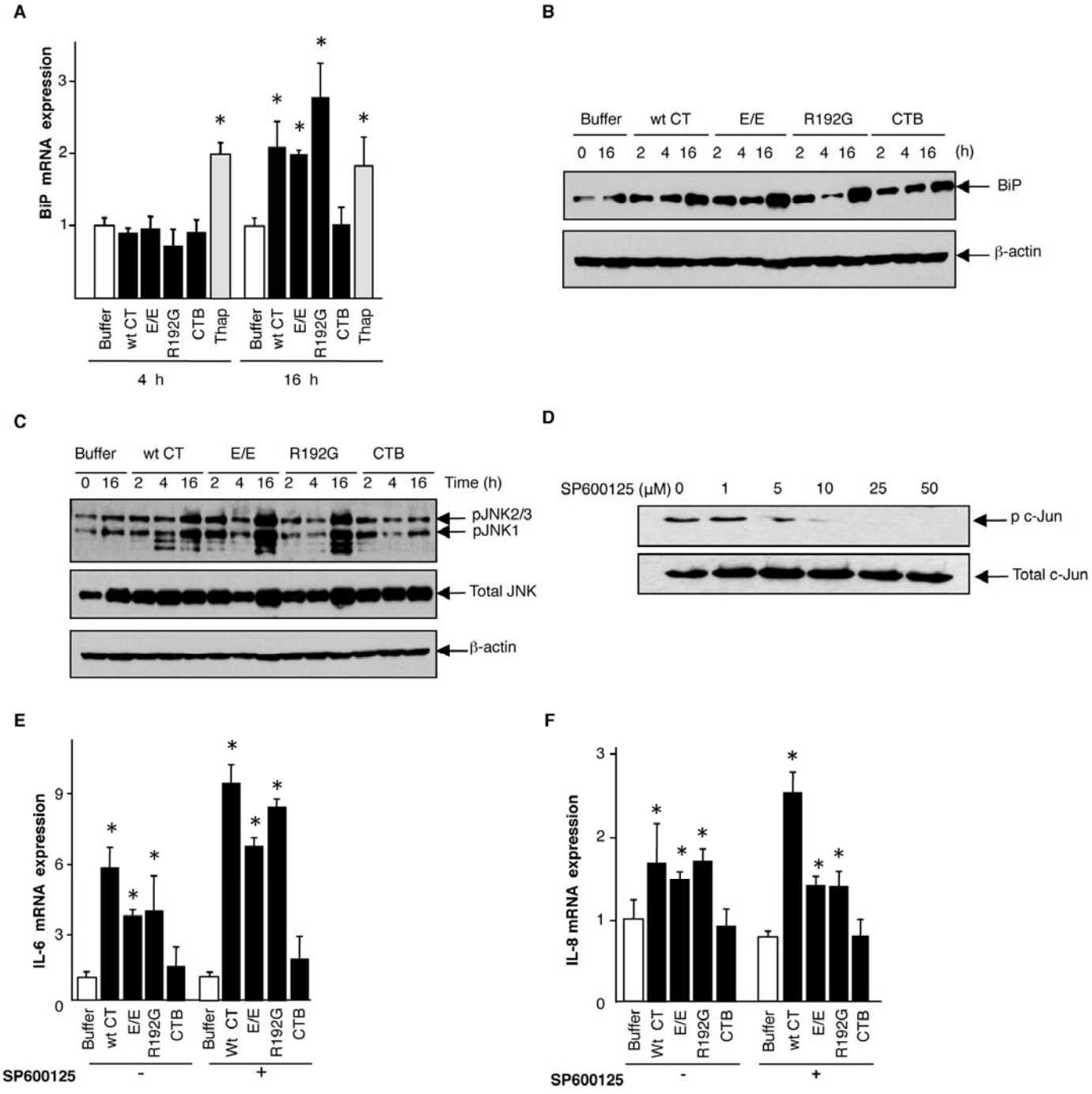

Figure S2. The A-subunit induces IL-6 and IL-8 independently of JNK and the ER chaperone BiP, Related to Figure 2

(A) Q-PCR analysis of BiP mRNA expression in polarized T84 cells intoxicated with 3 nM toxins for indicated times.

(B) Immunoblot for BiP in T84 total cell lysates intoxicated with 3 nM toxins for indicated times. β-actin is loading control.

(C) Immunoblot for phosphorylated c-Jun N-terminal kinase (JNK) in T84 cells. Total JNK and β-actin are loading controls. Phospho-JNK was apparent at 16 h after intoxication, but this is many hours after the induction of IL-6 and IL-8 cytokine transcription.
(D) Immunoblot for phosphorylated c-Jun, a downstream molecule of JNK, in T84 cells pretreated with different doses of SP600125, a selective inhibitor of JNK, for 30 min prior to the 4 h intoxication with 3 nM wt CT. Total c-Jun as loading control.
(E-F) Q-PCR analysis of IL-6 (E) and IL-8 (F) mRNA expression in polarized T84 cells pretreated with 10 μM SP600125 and intoxicated with 3nM wt or mutant toxins for 4 h. The inhibition of JNK activation by 10 μM SP600125 does not inhibit induction of IL-6 or IL-8 transcription by any of the holotoxins. CTB was inactive. β-actin is loading control.
Data are means ± SEM. *$p<0.05$, Nomenclature as described in Figure S1.

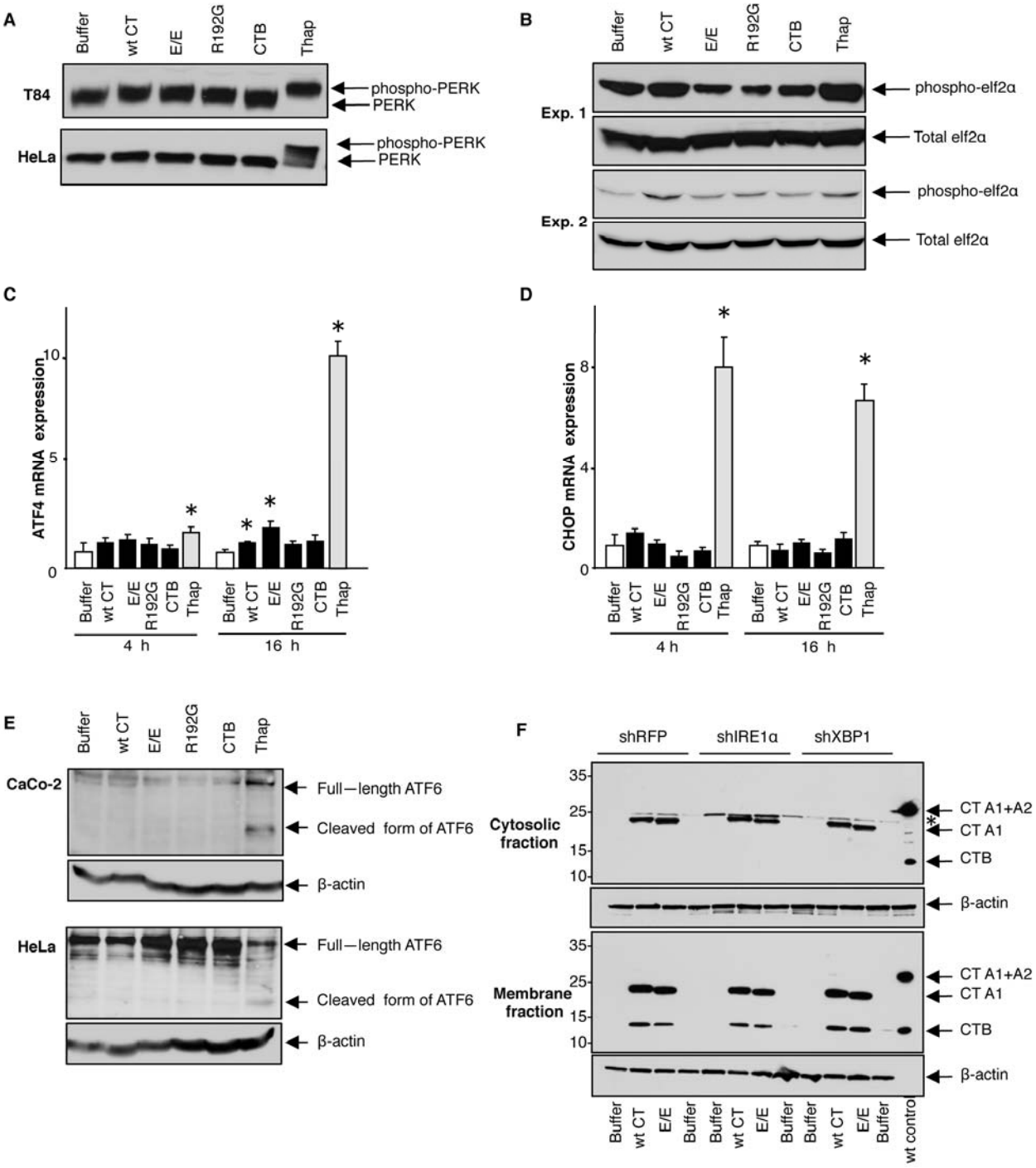

Figure S3. The A1 chain does not detectably activate the PERK or ATF6 pathways to induce inflammatory cytokines, and processing of the A-subunit by ERAD for retro-translocation does not depend on IRE1α or XBP1, Related to Figure 3

(A) Immunoblot for phospho-PERK using phos-tag SDS-PAGE in polarized T84 cells and HeLa cells intoxicated with 3 nM toxins for 16h. There was no detectable phosphorylation of PERK after 16 h of intoxication.
(B) Immunoblot for phosphorylated translation initiation factor (elf2α) in polarized T84 cells intoxicated with 3 nM CT for 16h.
(C-D) Q-PCR analysis of mRNA expression of ATF4 (C) and CHOP (D), the down-stream effectors of PERK activation, in T84 cells incubated with toxins for the indicated times. There were no detectable levels of ATF4 mRNA or CHOP mRNA after 4h of intoxication. At 16 h after intoxication, there is a small signal for expression of ATF4, many hours after induction of transcription of inflammatory cytokines, and consistent with the induction of the general UPR (BiP and ERdj4) at late times after toxin treatment.
(E) Immunoblot for ATF6 in the total lysate of polarized Caco-2 cells and HeLa cells intoxicated with 3 nM toxins for 16h. Activation of ATF6 was assessed by analyzing cleavage of the protein into its active fragments. ATF6 is not activated by the CT A-subunit.
(F) Immunoblot for CT A1-chain retro-translocation in stably transfected HeLa cells with shRNA against RFP, IRE1α or XBP1. Cells were intoxicated with 40 nM CT for 90 min followed by analysis. The membrane fraction was loaded at 10% volume of the cytosolic fraction. β-actin is the loading control. The A1-chain of the wt CT and the E/E mutant was detected equally in cytosolic fractions of cells lacking either IRE1α or XBP1. *: non-specific band,
Data are means ± SEM. *$p<0.05$. Nomenclature as described in Figure S1.

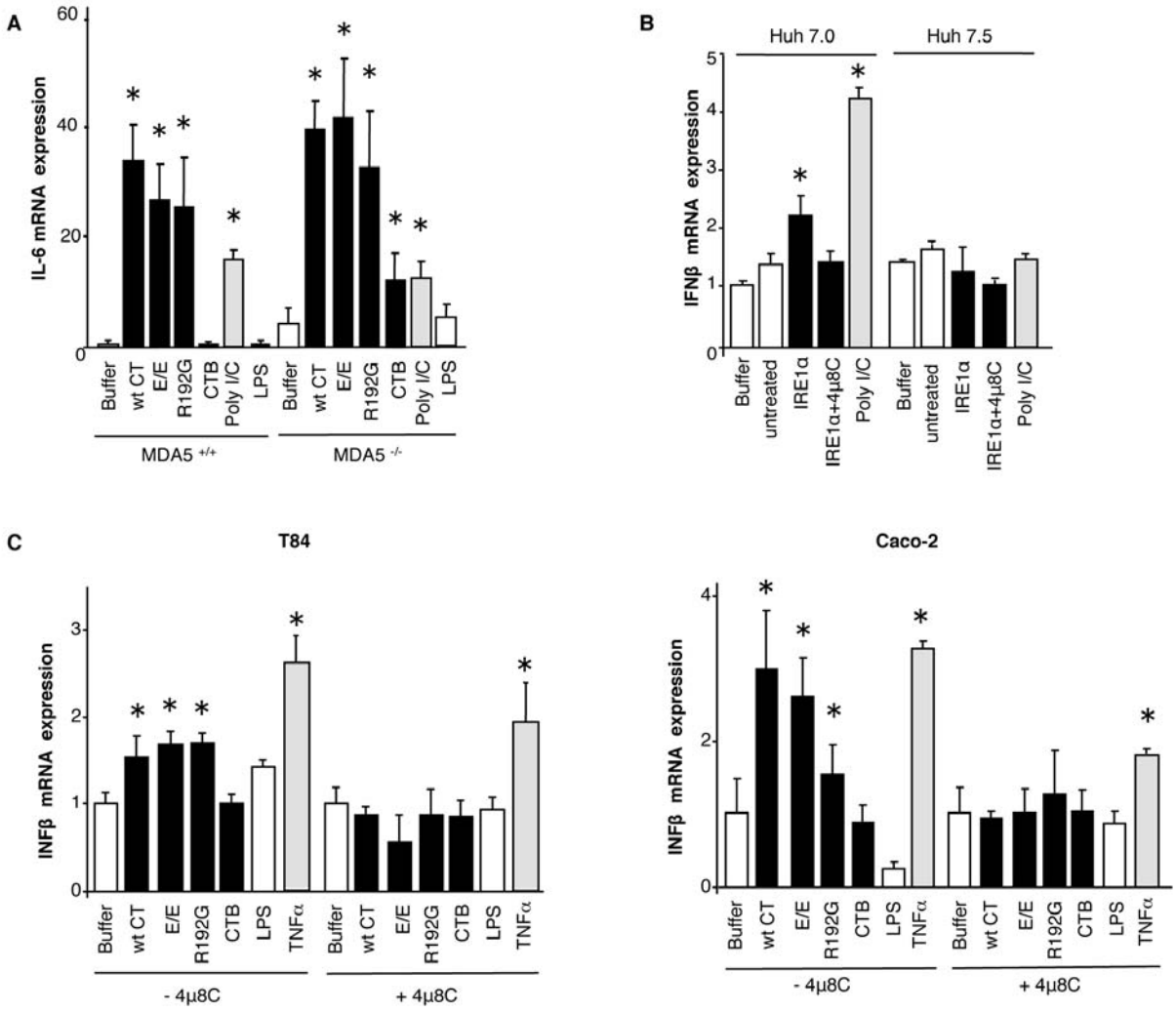

Figure S4. IRE1α activation and RIG-I activation by CT is independent of MDA5 and dependent on IFNβ induction, Related to Figure 4

(A) Q-PCR analysis of IL-6 mRNA expression in wt ($MDA5^{+/+}$) and MDA5 knockout ($MDA5^{-/-}$) MEF cells intoxicated with toxins for 4 h.

(B) Q-PCR analysis of IFN-β mRNA expression in Huh 7.0 (wt) and Huh 7.5 (mutant RIG-I) cells transfected with low molecular weight fragments of mRNA.

(C) Q-PCR analysis of IFN-β mRNA expression in polarized T84 cells or polarized Caco-2 cells pretreated with 5μM 4μ8C for 5 min followed by incubation with toxins for 4h. 4μ8C inhibited IFN-β mRNA induction by the CT-A1 chain. Data are means ± SEM. *$p<0.05$. Nomenclature as described in Figure S1.

Supplemental Experimental Procedures

Reagents and antibodies. Rabbit polyclonal anti-sera against CTB and CTA were prepared as previously described (Lencer et al., 1995). Antibodies (Ab) against JNK, phospho-JNK, c-Jun, phospho-c-Jun, CREB, phospho-CREB, PERK, IRE1α, phospho-elf2α, elf2α, p65, IKKα/β, phospho-IKKα/β, IκBα, and phospho-IκBα were purchased from Cell Signaling (Danvers, MA). Anti-TATA binding protein (TBP) Ab was from Abcam (Cambridge, MA). Goat anti-BiP/GRP78 and XBP1 Ab were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit ATF6α antibody was purchased from Rockland Immunochemicals Inc. (Gilbertsville, PA). The mouse Ab against β-actin was purchased from Sigma (St. Louis, MO). Secondary antibodies goat anti-rabbit immunoglobin G (IgG) (Sigma), goat anti-mouse IgG (Sigma), and donkey anti-goat IgG conjugated HRP (Santa Cruz), were used. Brefeldin A, thapsigargin, tunicamycin, and JNK inhibitor SP600125 were purchased from Sigma. Human TNFα was purchased from Cell Signaling. Bay 11-7082 was purchased from Enzo Life Sciences (Farmingdale, NY) and LPS was purchased from InvivoGen (San Diego, CA). 4μ8C is as described previously (Cross et al., 2012). Shiga-like toxin 1 was purchased from Tufts Medical Center (Boston, MA). SV40 is a generous gift from James DeCaprio (Dana Farber Cancer Institute, Boston, MA). Anthrax-fused Diphtheria is a generous gift from John Collier (Harvard Univeristy, Boston, MA).

Plasmids. E110D/E112D mutations were introduced into the arabinose-inducible pARCT (Wernick et al., 2010). The non-cleavable mutant (R192G), wild type CT (wt CT) and CTB have been previously described (Tsai et al., 2001). Lentiviral vectors, pLKO.1, containing shRNA targeting IRE1α and XBP1 were obtained from the Broad Institute (Cambridge, MA).

Purification of recombinant protein. Cholera toxins were purified as previously described (Wernick et al., 2010). Further purification was done over an ion exchange column in order to harvest the single intact form of the toxin and to remove *E. coli* contaminants including LPS. The core lumenal domain of murine Ire1α (aa: 26-392) was cloned into the pGEX 6P-2 plasmid and purified as an N-terminal GST fusion protein. Briefly, BL21 Star (DE3) *E. coli* carrying the mIre1α expression plasmid were induced at an OD600 of 0.7 with 0.3 mM IPTG for 16 hours at 18°C. Cells were harvested at 6000xg for 10 minutes and resuspended in lysis buffer (50 mM Tris pH 7, 10% glycerol, 150 mM NaCl, 4 mM DTT). Cells were lysed with 2 passages through the Avestin EmulsiFlex-C3 at 15,000 psi, and the supernatant was collected after 30 minutes at 30,000xg. The supernatant was batch bound to pre-washed glutathione sepharose for 1 hour, then poured into a column and washed with 300 mL lysis buffer. GST-mIre1α was eluted with 20 mM glutathione, further purified using a Superdex200 10/300 gel filtration column equilibrated with 50 mM Tris pH 7, 150 mM NaCl, 10% glycerol, 2 mM DTT and used for the peptide array.

Lentiviral transduction, stable cell line generation and transfection. Lentiviruses were produced by transfecting HEK293T cells with pLKO.1 shRNA vectors together with pCMV-ΔR8.9 and VSV-G packaging plasmids. Culture supernatants containing virus particles were collected 48 h after transfection and used to infect HeLa cells in the presence of 8 μg/ml polybrene. Transfected HeLa cells were selected in media containing 2 μg/ml puromycin. Transfection of RNA was done with lipofectamine 2000 (Invitrogen, Carlsbad, CA). Huh cells ($2X10^5$ per well in six-well plates) were transfected with 16 μl of low molecular weight RNA for 16 h.

Cytometric bead array. Polarized T84 cells in serum-free media were incubated with toxin for 4h. Cell media was then supplemented with serum and cells were further incubated 24-48 hours followed by cytokine analysis of the apical media using cytometric bead array kit (eBioscience, San Jose, CA).

Electrophysiology. Short-circuit current (I_{sc}) and resistance measurements in electrophysiological studies on polarized T84 monolayer (0.33 cm^2 inserts) were performed as previously described (Lencer et al., 1992). All measurements are representative of at least 3 independent experiments.

Cholera toxin retro-translocation assay. Retro-translocation experiments were modified from those previously described (Wernick et al., 2010). HeLa cells were plated at a concentration of 8×10^4 cells/well into 6-well plates and incubated for 45 min with 40 nM toxin in Hanks' Balanced Salt Solution (HBSS) supplemented with 10 mM HEPES pH 7.4. Cells were then washed in HBSS three times, detached with 0.25% trypsin with EDTA, pelleted by centrifugation and resuspended in HN buffer (50 mM HEPES, pH 7.5, 150 mM NaCl) supplemented with 0.5mg/ml EDTA and EDTA-free protease inhibitor tablets. After incubation on ice for 10 min cells were centrifuged at 80,000 rpm for 10 min at 4^oC in a TLA 100 rotor (Beckman Coulter). The supernatants were collected immediately as the cytosolic fraction and the membrane fraction pellet was resuspended in a volume of HN buffer equal to that of the cytosolic fraction. The protein concentration of the cytosolic fraction was measured by BCA. Equal amounts of the cytosolic fraction (40 μg) or the membrane fraction (4.5 μg) were loaded on SDS-PAGE and analyzed by immunoblot using the rabbit polyclonal antibody against CTA.

Supplemental References

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