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Supplementary Information
Necroptosis activates UPR sensors without disrupting their binding with GRP78.

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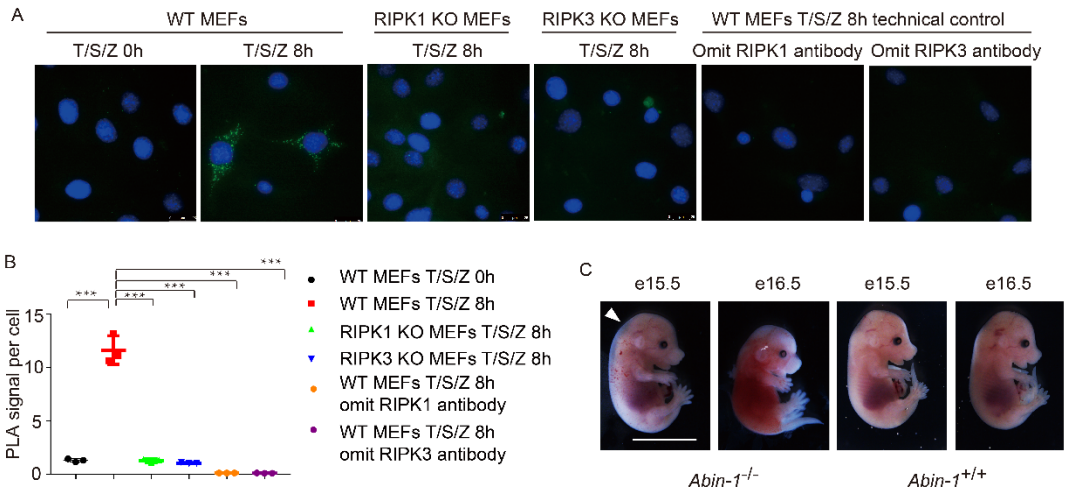

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Figure S1. Detection of RIPK1-RIPK3 necrosome in situ by PLA. (A) MEFs cells were treated with mouse TNF (10ng/ml), SM-164(100nM), and z-VAD.fmk(25μM) as indicated. The RIPK1-RIPK3 complexes were detected by in situ PLA. Scale bar,25 μm. (B) RIPK1-RIPK3 in situ PLA signals per cell were quantified (>100 cells per group from 3 independent experiment were assessed). (C) Representative wide-field images of Abin-$1^{+/+}$ and Abin-$1^{-/-}$ e15.5 and e16.5 embryos. The white arrow indicated the edema of Abin-$1^{-/-}$ embryo. Scale bar,1cm. All panels data is shown as mean ± s.e.m.; *p < 0.05, **p < 0.01, and ***p < 0.001, based on One-way ANOVA followed Tukey's test (B).

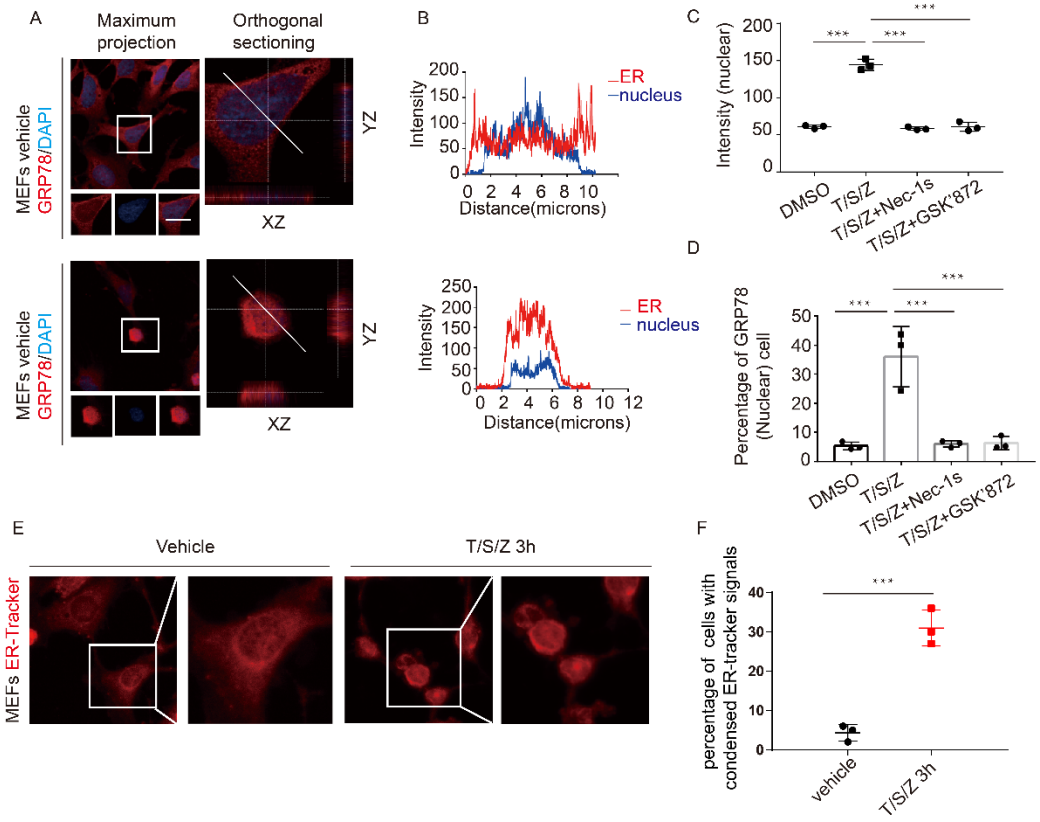

Figure S2. Association of necrosome with ER during necroptosis. (A) MEFs cells treated with mouse TNF(10ng/ml), SM-164(100nM), z-VAD.fmk(25μM), and ER was labeled by GRP78 immunofluorescence staining. Representative images of maximum projection and orthogonal sectioning. Scale bars,10 μm. (B) Quantification of the intensity along the line showed in (A). (C) Quantification of the average nuclear intensity. (D) Quantification of the percentage of cells with nuclear GRP78 (>100 cells per group from 3 independent experiment were assessed). (E-F) MEFs cells treated with mouse TNF (10ng/ml), SM-164(100nM), and z-VAD.fmk(25μM), as indicated and the ER was labeled by ER-Tracker red dye (E). Scale bar, 10 μm. Quantification of the percentage of cells with condensed ER-tracker signal (>100 cells per group from 3 independent experiment were assessed) (F). All panels data is shown as mean ± s.e.m.; *p < 0.05, **p < 0.01, and ***p < 0.001, based on One-way ANOVA followed Tukey's test (C, D) and two-tailed t-Student's test(F).

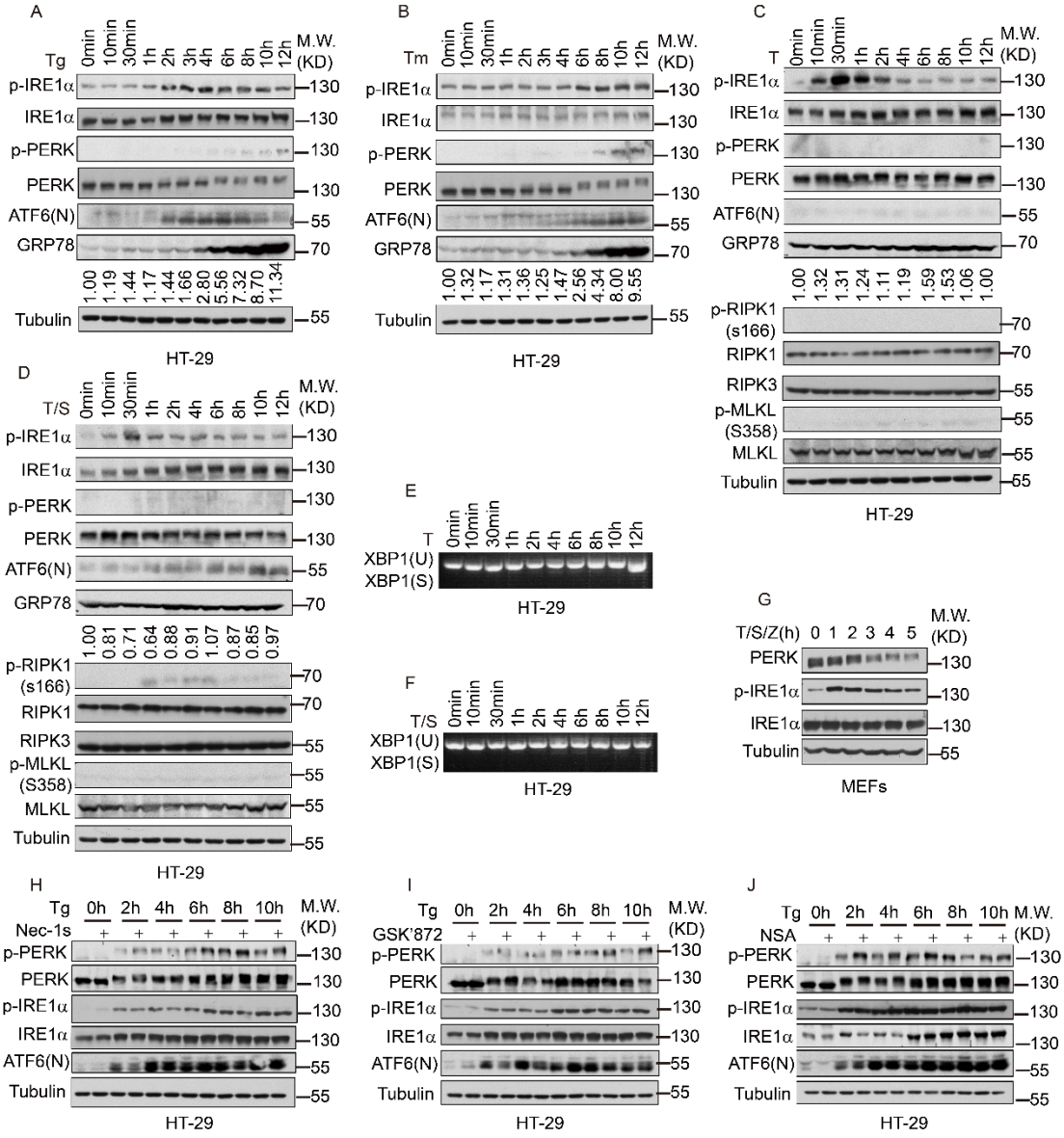

Figure S3. Necroptosis activates a non-canonical ER stress response. (A-F) HT-29 cells were treated with human TNF (50ng/ml), SM-164(100nM), Thapsigargin(Tg)(500nM), Tunicamycin(Tm)(1μg/ml) as indicated. The cell lysate was collected and analyzed by immunoblotting (A-D). The cell lysate was collected and analyzed by RT-PCR (E-F). (G) MEFs were treated mouse TNF (10ng/ml), SM-164(100nM). The cell lysate was collected and analyzed by immunoblotting. (H-J) HT-29 cells were treated with Thapsigargin(500nM), Nec-1s(10μM), GSK'872(10μM), and NSA(2.5μM) as indicated. The cell lysate was collected and analyzed by immunoblotting. The values under the GRP78 blots indicated quantification of relative gray value of GRP78 bands (A-D).

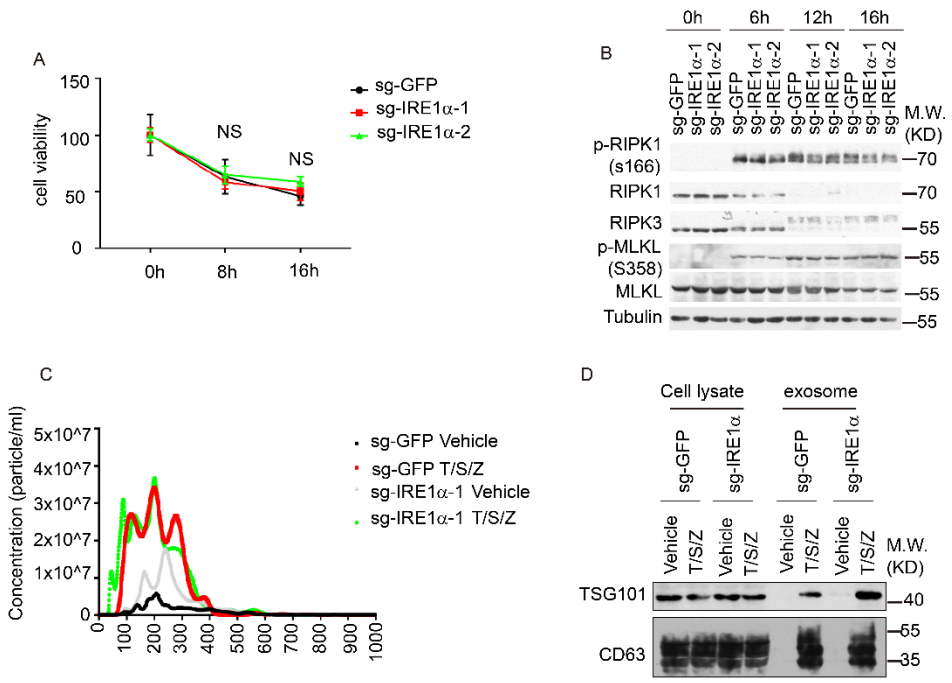

Figure S4. Incorporation of spliced XBP1 mRNA into extracellular vesicle during necroptosis. (A-B) Sg-GFP, sg-IRE1α-1, sg-IRE1α-2 HT-29 cells were treated with human TNF (50ng/ml), SM-164(100nM), and z-VAD.fmk(25μM) and the cell viability was determined by Cell-Titer-Glo assay following the treatment (A). The cell lysate was collected and analyzed by immunoblotting (B). (C-D) HT-29 cells were treated with human TNF (50ng/ml), SM-164(100nM), z-VAD.fmk (25μM) for 24h. The exosome was isolated from culture medium and analyzed by nanoparticle tracking analysis (NTA) (C). The exosome was isolated from culture medium and analyzed by immunoblotting (D). All data is shown as mean ± s.e.m.; *p < 0.05, **p < 0.01, and ***p < 0.001, based on two-way ANOVA followed by Bonferroni's test(A).

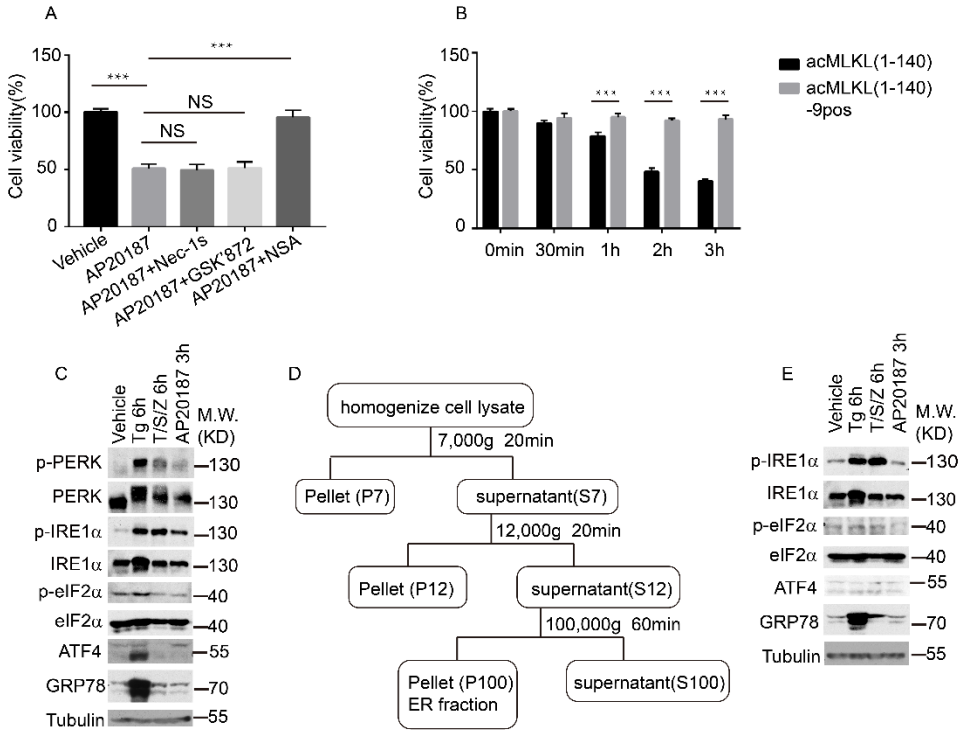

Figure S5. Oligomerized MLKL can activate UPR sensors without disrupting their binding with GRP78. (A-B) acMLKL HT-29 cells, acMLKL (1-140) HT-29 cells and acMLKL (1-140)-9pos cells were treated with AP20187(100nM), Nec-1s(10μM), GSK'872(10μM), NSA(2.5μM) as indicated and the viability was determined by Cell-Titer-Glo assay following the treatment. (C) acMLKL HT-29 cells were treated with thapsigargin(500nM), human TNF (50ng/ml), SM-164(100nM), z-VAD.fmk(25μM), AP20187(100nM) as indicated. The cell lysate was collected and analyzed by immunoblotting. (D) Schematic representation of ER isolation protocol. (E) PERK KO HT-29 cells reconstituted with dLD-PERK were treated with thapsigargin(500nM) or human TNF (50ng/ml), SM-164(100nM), z-VAD.fmk(25μM), Nec-1s(10μM) as indicated. The cell lysate was collected and analyzed by immunoblotting. All data is shown as mean ± s.e.m.; *p < 0.05, **p < 0.01, and ***p < 0.001, based on One-way ANOVA followed Tukey's test (A) and two-way ANOVA followed Bonferroni's test(B).

Supplemental Materials and Methods

Animals
Mating of male and female Abin-1 $^{+/-}$ mice at the age of 8-week were used to generate Abin-1$^{-/-}$ embryos.

Mouse husbandry
Special pathogen free (SPF) mice (C57BL/6) were used in this study. The mouse experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Institute of Interdisciplinary Research Center on Biology and Chemistry and performed according to the committee's guidelines.

Cell culture and treatments
Cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2. HT-29 cells were cultured in McCoy's 5A medium (GIBCO). MEFs and HEK293T cells were cultured in DMEM (GIBCO). Jurkat cells were cultured in RPMI 1640 medium (GIBCO). The culture medium was all supplemented with 10% FBS (GIBCO) and 100U/ml penicillin and 100U/ml streptomycin. For HT-29 cells human TNF was used at the concentration of 50 ng/ml. For MEFs cells 10ng/ml mouse TNF was used. SM-164, Nec-1s, GSK'872, NSA, z-VAD.fmk and AP20187 were used at 100nM, 10 μM, 20 μM, 2.5 μM, 25 μM, and 100 nM, respectively. Thapsigargin and Tunicamycin were treated by 500nM and 1μg/ml respectively.

Plasmid construction
The MLKL (1-140)-9pos DNA fragment was cloned by site-directed mutagenesis with KOD polymerase (Takara Bio). The DNA fragments of MLKL (aa 1-140, wild-type or mutant) and F36V FKBP were fused together into pMSCV vector by homologous recombination (Vazyme). The DNA fragment of FL-PERK and dLD-PERK were generated from the human cDNA by PCR, and cloned into pMSCV vector. The paired sgRNA oligos were annealed and cloned into the LentiCRISPR v2 vector (one vector system) cut by BsmBI. The paired shRNA oligos were annealed and cloned into the PLKO.1 vector cut by AgeI and EcoRI. All constructs were verified by sequencing. The target sequences of shRNA and sgRNA were listed below. human shRIPK3: 5'GGC -GACCGCTCGTTAACATAT3'. human shMLKL: 5'GAGT CAAATCTACAGCATATCG3'. human sgIRE1α-1: 5'CACCGCATGTTTGACAACCGCGACG3'. human sgIRE1α-2: 5'CACCGCTGCGAAGCTAAAACACTCG3'.

Virus packaging and transduction
Constructs and packing plasmids were co-transfected in to HEK 293T cells by polyethylenimine (PEI). The culture medium was filtered through the PVDF membrane (0.45μm) to collect the virus after 48 hours. Polybrene (8μg/ml) was added into the medium with virus, and HT-29 cells or MEFs were incubated with the mixture for 48h. The stable cell lines were selected with puromycin (2.5 μg/ml).

Reverse transcription PCR (RT-PCR) analysis
Total RNA was extracted by TRIzol reagent (Thermo Fisher Scientific). The cDNA was generated with the M-MLV reverse transcriptase (Takara Bio). Both the unspliced form and spliced form of XBP1 mRNA were detected by PCR. The primers for PCR were listed below. XBP1-F: 5'TTACGAGAGAAAACTCATGGCC3'. XBP1-R: 5'GGGTCCAAGTTGTCCAGAATGC3'. GAPDH-F: 5'TTGCCATCAATGACCC- CTTCA3'. GAPDH-R: 5'CGCCCCACTTGATTTTGGA3'.

Immunoprecipitation
Cell lysates were collected on ice with NP-40 buffer. The NP40 lysis buffer: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP40, 10% Glycerol, 5mM NaF, 1mM Na_3VO_4, 10mM β-glycerophosphate. PMSF(1mM) and 1x protease inhibitor cocktail (Biotool) were added into the buffer immediately before use. The supernatant was isolated by centrifuging at 15,000×*g* for 15 min at 4 °C. The antibodies of interest were added into the supernatant followed by incubation at 4 °C overnight. The Protein G Agarose (Thermo Fisher Scientific) was added into the supernatant on the next day followed by incubation at 4 °C for 4h. The supernatant was removed from the agarose beads by centrifuging and washing 5 times with NP40 lysis buffer. The SDS loading buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, and 100 mM DTT) was added into the beads and the mixture was boiled for 10 min. The supernatant isolated by centrifuge was used for subsequent analysis.

Western blotting
Cell lysates were electrophoresed by SDS-PAGE. The gels were transferred onto nitrocellulose membranes (GE healthcare). The membrane was blocked with 5%(w/v) non-fat dry milk in 0.1% Tween-20 Tris-buffered saline (TBST). The membrane was incubated with primary antibodies at 4 °C overnight and washed in TBST for 3 times. Then the membrane was incubated with the HRP-conjugated secondary antibodies at room tempreture for 1 hour and washed with TBST for 3 times. The blots were developed using the chemiluminescence substrate. Antibodies used in the western blotting analysis include: Anti-RIPK1 (Cat#3493), Anti-pRIPK1(s166)(human specific) (Cat#65746), Anti-PERK(Cat#5683), Anti-IRE1α (Cat# 3294), Anti-GRP78 (Cat#3177) and Anti-VDAC (Cat#4866) from Cell Signaling Technology; Anti-RIPK3(human) (Cat#ab72106), Anti-MLKL(human) (Cat#ab183770) and Anti-pMLKL(T357/S358) (Cat#ab187091) from Abcam; Anti-pPERK(T982) (PA5-102853) from Invitrogen; Anti-p-IRE1α(S724) (Cat#NB100-2323) from Novus; Anti-ATF6 (Cat#24169-1-AP) and Anti-Calnexin (Cat#10427-2-AP) and from PTG; Anti-α-Tubulin (Cat#PM054) and Anti-β-Actin (Cat#PM053) from MBL; TSG101 (C-2) (Cat#sc-7964) and CD63 (MX-49.129.5) (Cat#sc-5275) from Santa Cruz; Histone H2AX (Cat#A11463) from Abclonal; HRP conjugated goat anti-rabbit IgG (H+L) secondary antibody (Cat#31460) and HRP conjugated goat anti-mouse IgG (H+L) secondary antibody (Cat#31430) from Thermo Fisher Scientific.

Viability assay
Cell viability was assayed by the CellTiter-Glo Luminescent Cell Viability Assay (Cat#G7572) from Promega. All cell viability assays were performed with triplicates.

Immunofluorescence
Cells were seeded on coverslips. After treatment as indicated, the cells were fixed with 4% paraformaldehyde and the PFA was washed out by PBS for 3 times. Then cells were permeabilized with 0.25% Triton X-100 in PBS and blocked with 5% bovine serum albumin (BSA) in PBS for 1h. Cells were incubated with the primary antibody overnight at 4 °C and washed by PBS for 3 times. Then cells were incubated with the fluorescent secondary antibody for 1h at room temperature and washed by PBS for 3 times. Cells were mounted with ProLong™ Diamond Antifade Mountant (Invitrogen) and images were captured (Leica SP8 Confocal System). Antibodies used in immunofluorescence assay were: Anti-Cox IV (Cat#ab33985), Anti-Calnexin (Cat#ab112995) and Anti-GRP78 (Cat#ab21685) from Abcam; Anti-EEA1(Cat#610457) and Anti-GM130 (Cat#610822) from BD Bioscience.

Collection and quantification of extracellular vesicles

The cells were cultured with the medium with 10%FBS. The medium was replaced with fresh medium without FBS immediately before stimulation. The medium was centrifuged successively at increasing speeds, 300g at 4°C for 10min, 2000g at 4°C for 10min, and 10,000g at 4°C for 10min. At each of these steps, the pellet was thrown away, and the supernatant was used for the following steps. Then the supernatant was centrifuged at 100,000g for 70min at 4°C, the pellet was washed by PBS and then centrifuged at 100,000g for 70min again. The pellet was collected and stored at -80 °C before use. Size spectra and particle number of the extracellular vesicles were determined by Nanoparticle Tracking Analysis (NTA) using the NanoSight NS300 device (Malvern Instruments) according to the manufacturer's instructions.

Isolation of ER

Instruction of Endoplasmic Reticulum Isolation Kit (Cat#ER0100) was followed with minor modifications. Briefly, FADD deficient Jurkat cells were harvested by centrifugation at 600g for 5 min. The cell pellets were washed by 10 volume of PBS and centrifuged at 600g for 5min again. Then cell pellets were re-suspended in 4 volumes of isotonic extraction buffer (10mM HEPEs, PH7.8, 250mM sucrose, 25mM potassium chloride, and 1mM EGTA, 1mM PMSF, 1x protease inhibitor cocktail). The cell lysate was homogenized by passing through a 22-G needle for 20 times. The cell lysate was centrifuged at 7000g for 20min. The supernatant was designated as S7. The pellet was re-suspended in the same volume of isotonic extraction buffer and designated as the P7 fraction. The supernatant (S7) was centrifuged at 12,000g for 20min. The supernatant was separated as S12. The pellet was re-suspended in the same volume of isotonic extraction buffer as P12 fraction. The supernatant (S12) was centrifuged at 100,000g for 1h and the supernatant was harvested as S100. The pellet was re-suspended in the same volume of isotonic extraction buffer as P100 fraction. All centrifugations were performed at 4°C unless otherwise indicated.