Figure S1

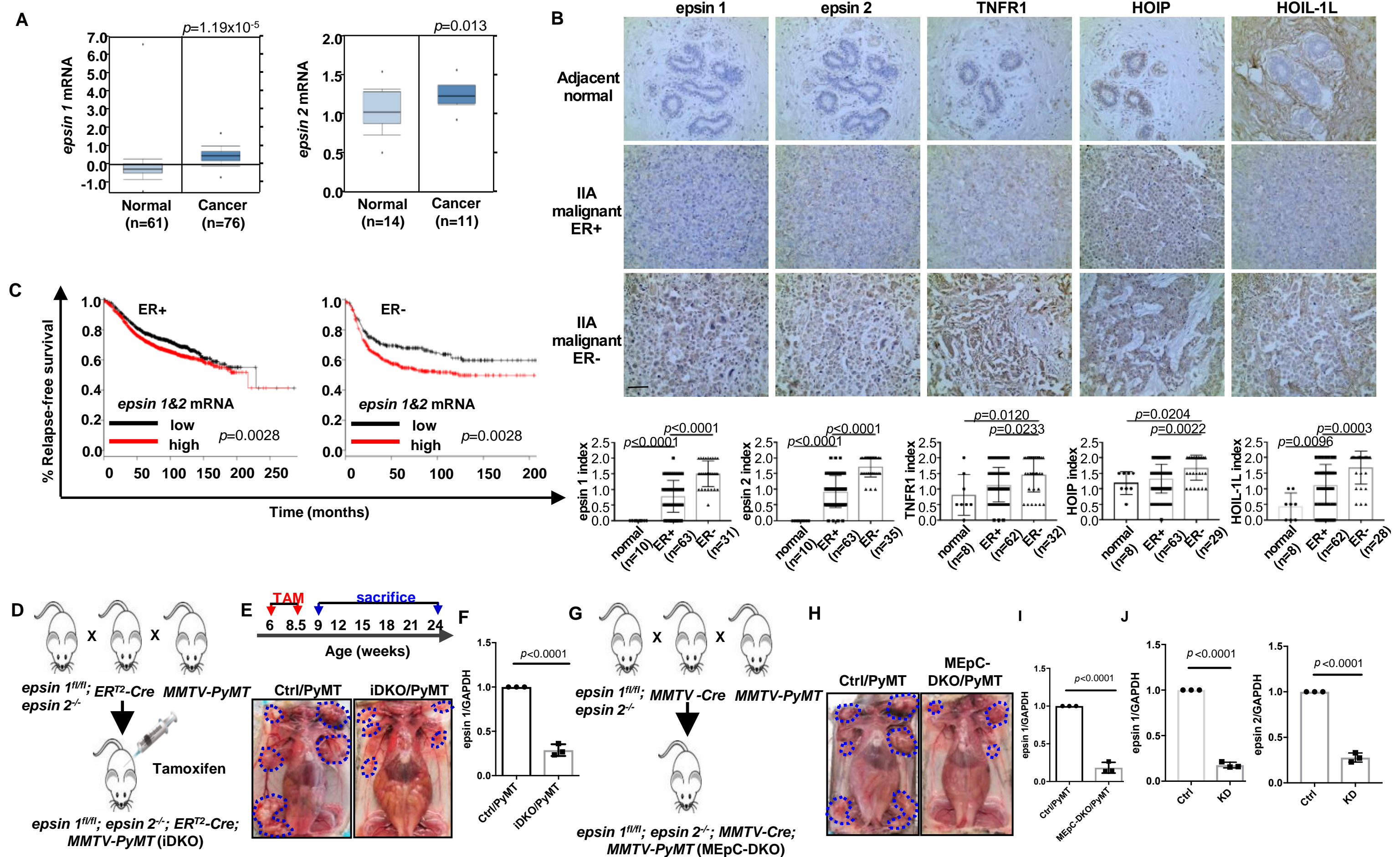

Figure S2

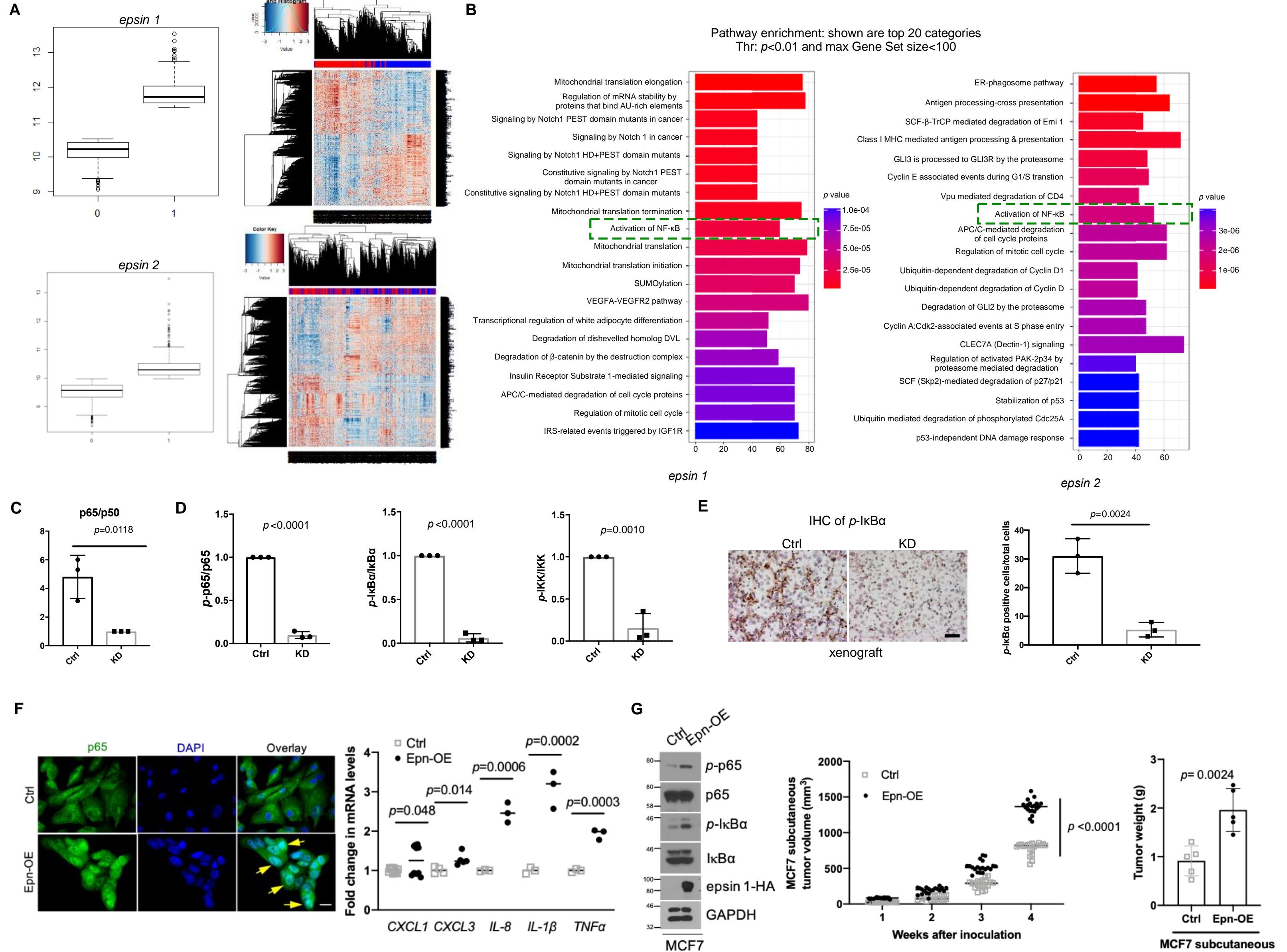

Figure S3

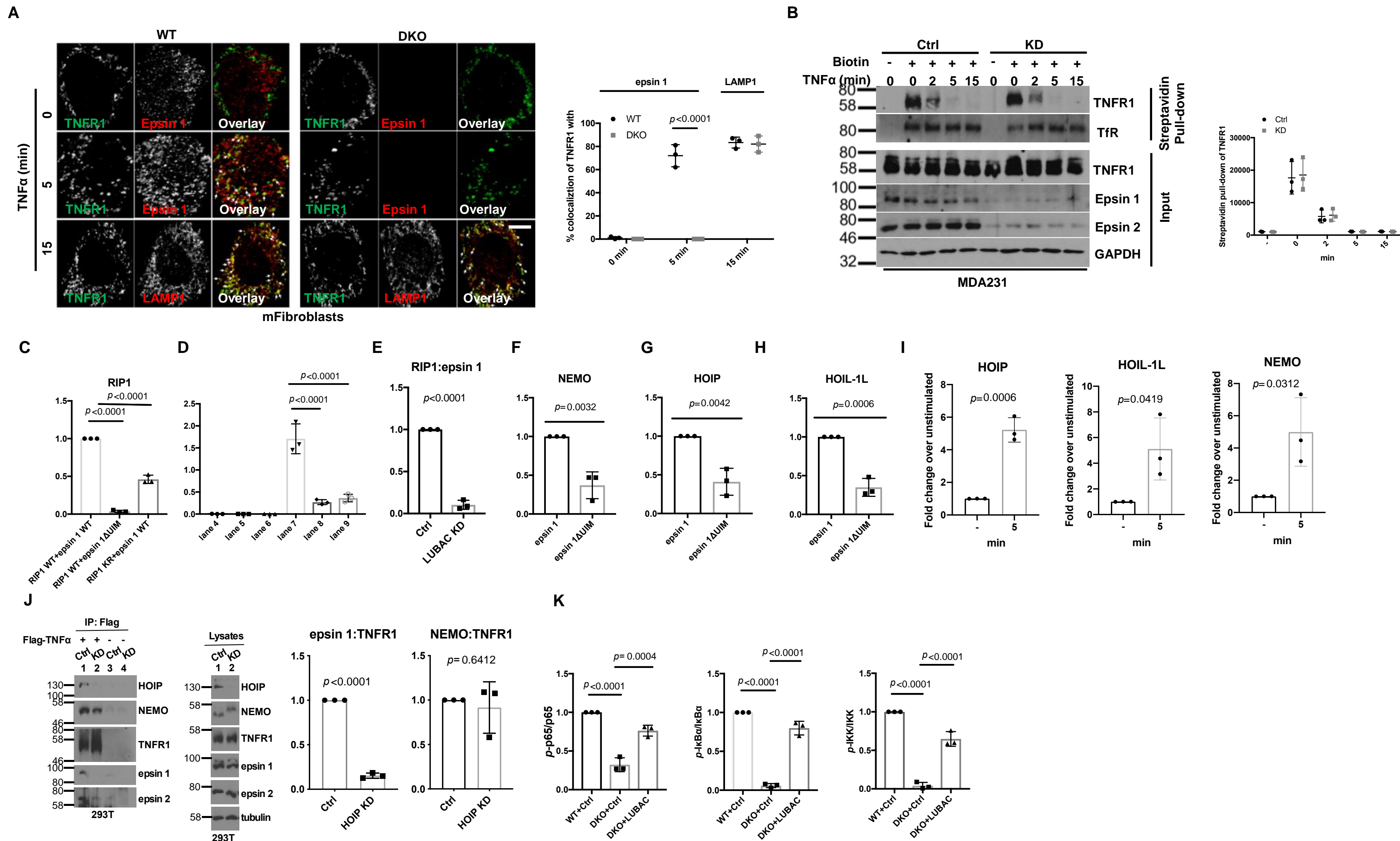

Figure S4

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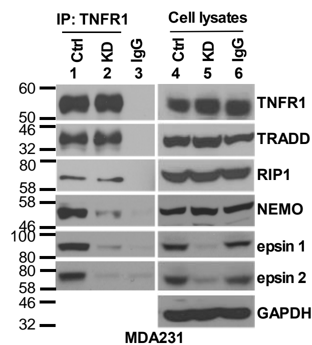

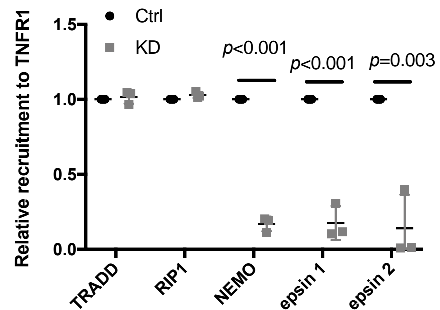

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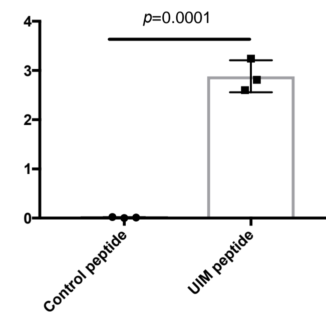

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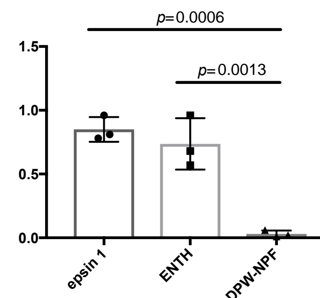

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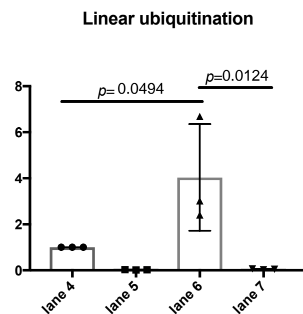

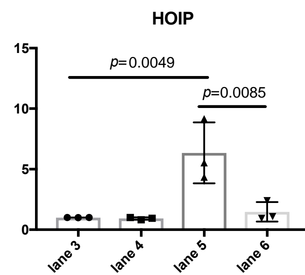

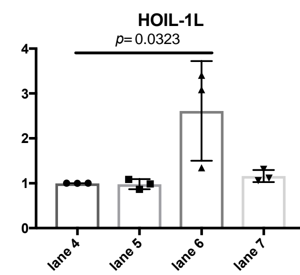

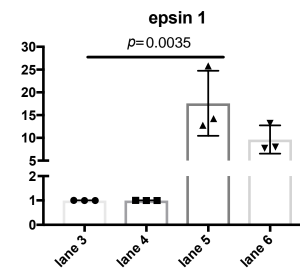

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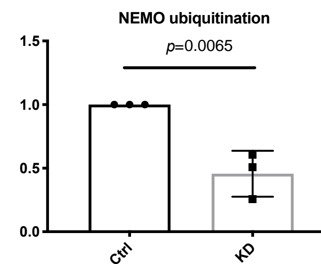
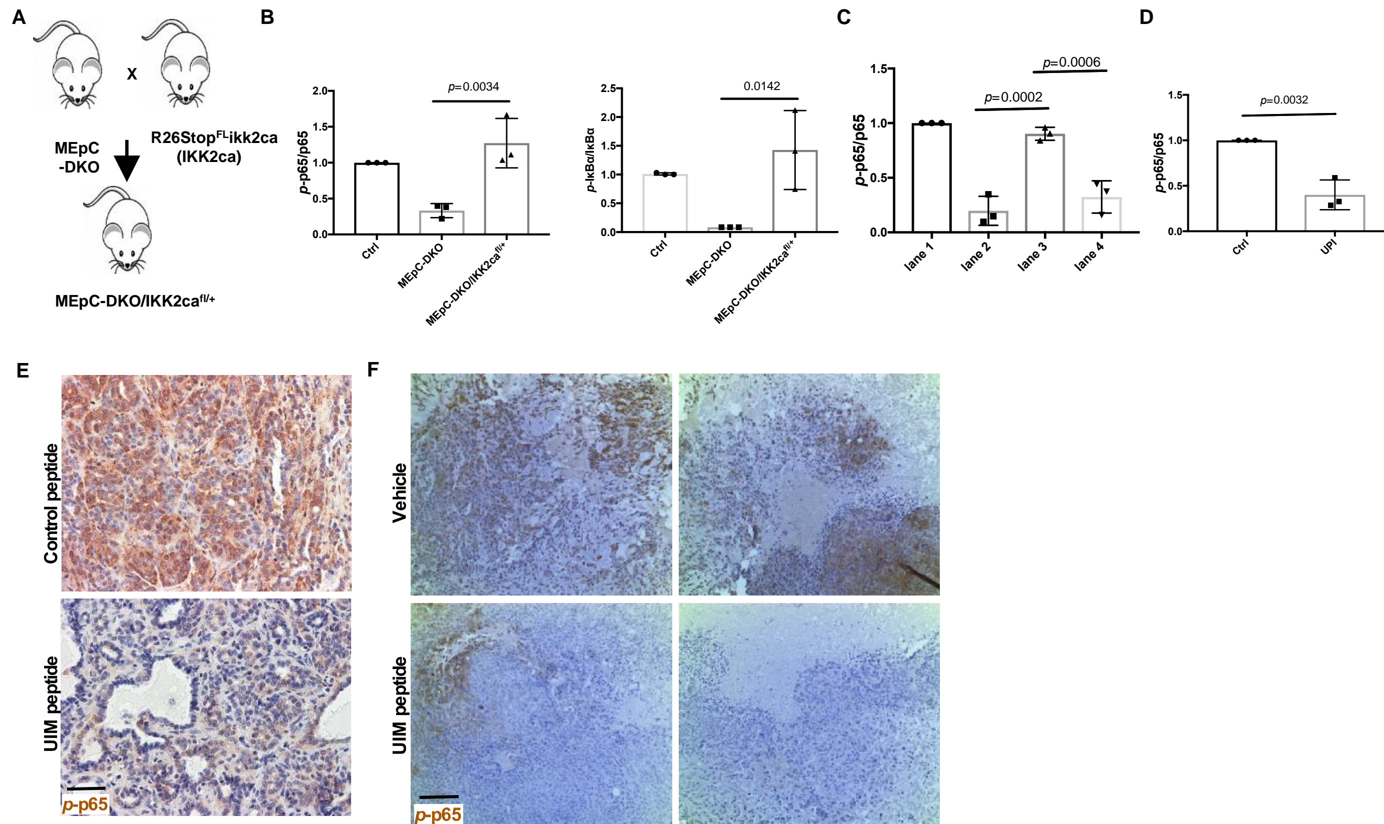


Figure S5



SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Epsins are upregulated in breast cancer and negatively correlate with relapse-free survival in breast cancer patients. (A) Microarray analyses of epsin 1 and epsin 2 mRNA expression from an Oncomine data set of normal and cancerous human breast tissue. **(B)** IHC analysis and quantification of epsin 1, epsin 2, TNFR1, HOIP, and HOIL-1L in human normal adjacent mammary tissues (n=10 biologically independent samples), ER-positive breast cancer (n=63 biologically independent samples), or ER-negative breast cancer (n=35 biologically independent samples) tumor specimens. Statistical values were calculated using one-way ANOVA followed by Tukey's multiple comparisons test. Data are presented as the mean ± SD. Scale bar: 50 μm. **(C)** Kaplan-Meier plot of relapse-free survival of patients stratified by median *EPN1/2* expression in different breast cancer subtypes from breast cancer meta-analysis database. Sample size is over 5000. **(D)** Mating schematic for generation of the global inducible epsin-deficient MMTV-PyMT model. **(E)** Gross analysis of tumor growth in tamoxifen-induced global epsin 1 and 2 double knock out (iDKO) MMTV-PyMT mice at 21 weeks of age compared to MMTV-PyMT control (Ctrl) mice. Circles indicate mammary tumors. **(F)** Western blot analysis of epsin 1 in Ctrl/PyMT and iDKO/PyMT tumor tissues. **(G)** Mating schematic for generation of the mammary epithelial epsin deficient MMTV-PyMT model (MEpC-DKO). **(H)** Representative image of gross primary mammary tumors indicated by circles in control and MEpC-DKO MMTV-PyMT mice at 21 weeks. **(I)** Western blotting analysis of epsin 1 in Ctrl and MEpC-DKO tumor tissues. **(J)** Western blotting analysis of epsins 1 and 2 knockdown in MDA231 cells infected with lentivirus expressing scrambled shRNA (Ctrl) or shRNA specific to epsins 1 and 2 (KD). Statistical values were calculated using one-way ANOVA followed by Tukey's multiple comparison test (**B**). Statistical values were calculated using an unpaired, two-tailed *t*-test (F, **I**, **J**). Data are presented as mean ± SD.

Figure S2. Pathways that correlate with epsin 1 and 2 expression in human breast cancer specimens. (A) TCGA BRCA level 3 (normalized) RNAseq gene expression and clinical data were downloaded from the Firehose source at the Broad Institute. The total number of samples was 1098. Differential gene expression was computed by a linear-design-model based estimation using the Limma package. The heatmap was

constructed on quantile ranked selection based on *EPN1 or EPN2* genes and selecting samples with low (<= 25%) and high (>= 75%) expression, which resulted in 548 samples. Furthermore, a threshold cutoff of a 1e-7 adjusted *p* value was used for probe selection, resulting in a final matrix of 548 samples and 8261 probe IDs. **(B)** The top 20 pathways that correlate with *EPN1* and/or *EPN2* expression in human breast cancer specimens, including Notch, VEGF, and Wnt signaling pathways, as previously reported. The data suggest that both *EPN1* and *EPN2* high populations are enriched for genes involved in NF-κB pathway activation. **(C)** Quantification of Fig. 2c. **(D)** Quantification of Fig. 2d. **(E)** IHC analysis of *p*-IκBα protein levels in primary orthotopically implanted Ctrl or KD MDA231 tumors from nude mice. Statistical values were calculated using an unpaired, two-tailed t-test (**C**, **D**, **E**). Data are presented as mean ± SD. **(F)** Confocal immunofluorescent imaging of p65 localization in Ctrl and epsin 1 overexpressing (Epn-OE) MCF10A cells. qRT-PCR analysis of NF-κB target genes *CXCL1*, *CXCL3*, *IL-8*, *IL-1β* and *TNFα* in Ctrl and Epn-OE MCF10A cells. **(G)** Growth curves and tumor weight of subcutaneously implanted Ctrl or Epn-OE MCF7 tumors in SCID mice. We generated MCF10A cells (**F**) and MCF7 cells (**G**) that stably overexpress LacZ or epsin 1 by infecting cells with lentivirus containing LacZ or epsin 1 cDNA (Ctrl or Epn-OE). Statistical values were calculated using an unpaired, two-tailed *t*-test (**F** and **G**). Data are presented as mean ± SD.

Figure S3. Epsins 1 and 2 regulate TNFR1 signaling without affecting TNFR1 endocytosis. **(A)** IF analysis of TNFR1 localization using biotinylated TNFα and indicated antibodies in primary fibroblasts isolated from WT and iDKO mice. **(B)** Western blot analysis of TNFα (20 ng/ml) induced loss of cell surface TNFR1 in Ctrl and KD MDA231 cells. These data show that endocytosis of TNFR1 occurs independently of epsins. **(C)** Quantification of Fig. 3B. **(D)** Quantification of Fig. 3C. **(E)** Quantification of Fig. 3D. **(F)** Quantification of Fig. 3E. **(G)** Quantification of Fig. 3F. **(H)** Quantification of Fig. 3G. **(I)** Quantification of Fig. 3H. (**J**) Western blot analysis and quantification of anti-Flag immunoprecipitation in control (generated by transfecting scrambled siRNA using Lipofectamine™ RNAiMAX, Ctrl) or HOIP knockdown (generated by transfecting siRNA against HOIP, KD) 293T cells, which were stimulated with Flag-TNFα (1 μg/ml) for 5 minutes. **(K)** Quantification of Fig. 3J. Statistical values were calculated using one-way ANOVA followed by Tukey's multiple

comparison test (**C**, **D**, **K**). Statistical values were calculated using an unpaired, two-tailed *t*-test (**A**, **B**, **E**, **F**, **G**, **H**, **I**, **J**). Data are presented as mean ± SD.

Figure S4. Epsins 1 and 2 constitutively associate with the TNFR1 complex in human breast cancer cells and the recruitment of NEMO was impaired upon epsin knockdown. **(A)** Anti-TNFR1 immunoprecipitation using lysates of control or epsin-deficient MDA231 cells was performed. Ctrl and KD MDA231 cells were lysed and pulled down using protein G Sepharose 4B conjugated with TNFR1 antibody at 4°C for 16 hours, following by western blot analysis of epsins, NEMO, and components of the TNF receptor signaling complex (TNF-RSC). The results suggest that epsins might play an important regulatory role in preventing the TNFR1 downstream IKK complex/signalosome from falling apart from the TNFR1 complex in breast cancer cells. **(B)** Quantification of Fig. 4A. **(C)** Quantification of Fig. 4B. **(D)** Quantification of Fig. 4C. **(E)** Quantification of Fig. 4D. Statistical values were calculated using an unpaired, two-tailed *t*-test (**A**, B, **E**). Statistical values were calculated using one-way ANOVA followed by Tukey's multiple comparison test (**C**, **D**). Data are presented as mean ± SD.

Figure S5. Epsin-mediated NF-κB activation is critical for breast cancer development. **(A)** Mating schematic for generation of MEpC-DKO mice with IKK2 constitutive active transgenic incorporation (MEpC-DKO/IKK2ca$^{fl/+}$). **(B)** Quantification of western blot analysis of *p*-p65 and *p*-IκBα in breast tumor tissues in control, MEpC-DKO or MEpC-DKO/IKK2ca$^{fl/+}$ MMTV-PyMT mice. **(C)** Quantification of Fig. 5C. **(D)** Quantification of Fig. 5D. Statistical values were calculated using one-way ANOVA followed by Tukey's multiple comparison test (**B**, **C**). Statistical values were calculated using an unpaired, two-tailed *t*-test (**D**). Data are presented as mean ± SD. (**E**) Representative images from IHC analysis of *p*-p65 in primary tumors from control or epsin UIM peptide treated MMTV-PyMT mice. (**F**) Representative images from IHC analysis of *p*-p65 in primary tumors from sham or epsin UIM peptide treated triple-negative breast cancer PDX model. Scale bars: 100 μm (**E**, **F**).

SUPPLEMENTAL INFORMATION

METHODS

Antibodies and Reagents

Unless specified otherwise, common laboratory chemicals and reagents were from either Sigma-Aldrich or Fisher Scientific. Media and additives for cell culture were from Gibco. Reagents and materials for RNA isolation, qRT-PCR, transfection reagents and primers were from Invitrogen. QuikChange II Site-Directed Mutagenesis Kit was from Agilent Technologies. 4-hydroxytamoxifen and Carmine Red were from Sigma-Aldrich. Recombinant human TNFα was from R&D systems. Polyclonal rabbit antibodies for epsins 1 and 2 were obtained as described[1, 2]. Anti-transferrin receptor (#13113), anti-IκBα (#4814S), anti-phospho-IKKβ/α (#2697), anti-IKKβ (#8943), anti-TRADD (#3684S), anti-p65 (#8242), anti-phospho-p65 (#3033S), anti-phospho-IκBα (#2859), anti-RIP1 (#3493S), anti-cleaved caspase 3 (#9679), anti-caspase 3 (#9662) were purchased from Cell Signaling Technology. Anti-NEMO (#ab178812) and anti-HOIP (#ab46322) were from Abcam. Anti-TNFR1 (#AF225) was from R&D Systems. anti-HOIL-1L (#MABC576) was from EMD Millipore. Anti-HA (#631207) was from Clontech Laboratories. Anti-Flag (#F1804-1MG) was from Sigma-Aldrich. Anti-Xpress (#R910-25) was from ThermoFisher Scientific. Anti-GAPDH (#sc-166545) was from Santa Cruz Biotechnologies.

Mice

All animal procedures were approved by Institutional Animal Care and Use Committee (IACUC) of Oklahoma Medical Research Foundation. We reported a strategy to generate an *epsin 1* tissue-specific and *epsin 2* global knockout mouse model[3, 4]. To create a mammary epithelial cell-specific knockout of *epsin 1* and a global knockout of *epsin 2* mouse model, we crossed *epsin 1*$^{fl/fl}$*; epsin 2*$^{-/-}$ mice with *MMTV-Cre* transgenic mice purchased from Jackson Laboratory (stock number 003553), which express Cre recombinase specifically in the mammary epithelial cells[5], and generated mammary epithelial-specific double knockout mice (MEpC-DKO). These mice were then backcrossed onto the C57BL/6 background. To generate the epithelial cell-specific epsins 1 and 2 double knockout spontaneous

breast cancer mouse model, we crossed MEpC-DKO mice with *MMTV-PyMT* transgenic mice (purchased from Jackson Laboratory, stock number 002374). Given that *MMTV-PyMT* mice are on the FVB genetic background, we backcrossed *MMTV-PyMT* mice onto the C57BL/6 background. We then bred the resultant mice with 1) WT C57BL/6 mice to obtain WT/PyMT mice; 2) MEpC-DKO mice to obtain *epsin 1*$^{fl/fl}$*; epsin 2*$^{-/-}$*; MMTV-PyMT; MMTV-Cre* mice (MEpC- DKO/PyMT mice on the C57BL/6 background). Given that epsins 1 and 2 play a superimposing role based on our previous study, for additional controls, we utilized *epsin 1*$^{+/+}$*; epsin 2*$^{-/-}$; *MMTV-PyMT; MMTV-Cre* and *epsin 1*$^{fl/fl}$*; epsin 2*$^{-/-}$*; MMTV-PyMT* littermates. The control mice cohort (Ctrl/PyMT) for MEpC-DKO/PyMT, consisting of both *epsin 1*$^{+/+}$*; epsin 2*$^{-/-}$; *MMTV-PyMT; MMTV-Cre* with a single copy of *MMTV-Cre*, and *epsin 1*$^{fl/fl}$*; epsin 2*$^{-/-}$*; MMTV-PyMT* littermate controls lacking the single copy of *MMTV-Cre*, exhibit no difference in phenotypes compared with WT/PyMT mice.

We previously reported an inducible global epsins 1 and 2 double knockout mouse model, tamoxifen-inducible *epsin 1*$^{fl/fl}$*; epsin 2*$^{-/-}$*; ER*^{T2}*-Cre* mice on the C57BL/6J background[4, 6]. Upon tamoxifen-induction the ER^{T2}-driven Cre recombinase is ubiquitously expressed. To generate the inducible global epsins 1 and 2 double knockout spontaneous breast cancer mouse model, we crossed *epsin 1*$^{fl/fl}$*; epsin 2*$^{-/-}$*; ER*^{T2}*-Cre* mice with *MMTV-PyMT* mice on the C57BL/6 background. The crosses resulted in *epsin 1*$^{fl/fl}$*; epsin 2*$^{-/-}$*; ER*^{T2}*-Cre*; *MMTV-PyMT* (iDKO/PyMT) mice. Similarly, for additional controls, we utilized *epsin 1*$^{+/+}$*; epsin 2*$^{-/-}$; *ER*^{T2}*-Cre*; *MMTV-PyMT* and *epsin 1*$^{fl/fl}$*; epsin 2*$^{-/-}$; *MMTV-PyMT* littermates, both of which exhibit no difference in phenotypes compared to WT/PyMT mice. To induce postnatal deletion, 4-hydroxytamoxifen was administered by intraperitoneal injection (150 μg per 30 g of body weight) at 6 weeks of age on alternative days for 2 weeks. To rule out the effect of 4- hydroxytamoxifen, we administered 4-hydroxytamoxifen to all animals including the Cre- negative controls.

To rescue defective NF-κB signaling, we crossed the MEpC-DKO mouse with the R26Stop^{FL}ikk2ca (*IKK2ca*) mouse[7-9] on the C57BL/6 background purchased from Jackson Laboratory (stock number 008242) to generate the MEpC-DKO/IKK2ca$^{fl/+}$ mouse. IKK2ca mouse harbors a constitutively activated IKK2ca$^{fl/+}$ transgene. Upon

exposing to Cre recombinase, a floxed stop codon was removed, leading to the expression of the constitutively active IKK2 transgene and subsequent NF-κB activation. MEpC-DKO/IKK2ca$^{fl/+}$ mouse was experimentally compared to MEpC-DKO littermates.

Cell Lines and Cell Culture

MCF7 (ATCC#HTB-22) and MDA-MB-231 (MDA231, ATCC#HTB-26) cells were originally purchased from American Type Culture Collection (ATCC) and cultured as previously described[10]. HMLE cells were a gift from Dr. Yibin Kang at Princeton University and cultured in DMEM/F12 (1:1) media containing 5% horse serum, 10 μg/ml insulin, 20 ng/nl EGF, 100 ng/ml cholera toxin and 500 ng/ml hydrocortisone. Primary mouse dermal fibroblast cultures were established by enzymatic digestion of the dermis as previously described[11].

Plasmids and Transfection

Flag-NEMO WT plasmid was a kind gift from Dr. Wang Min from Yale University. Flag- NEMO K285/309R double-mutant plasmid was generated using QuikChange Site- Directed Mutagenesis Kit per manufacturer's instruction. Cloning and mutagenesis primers are available upon request. Epsin 1, epsin 1 ΔUIM, ENTH, and DPW-NPF plasmids and lentiviral pLL3.7 RNA interference vectors encoding epsins 1 and 2 shRNA were described previously[2, 4, 12]. The retroviral pLEX plasmids were originally obtained from Open Biosystems. Other plasmids if not described were obtained from Addgene. HEK 293T cells were transfected using Lipofectamine 2000 as instructed by the manufacturer. MDA231 cells and mouse primary fibroblasts were transfected using Lipofectamine LTX with Plus™ reagent as instructed by the manufacturer. Lentiviral production was accomplished by transfecting Phoenix ECO cells (ATCC#CRL-3214) using Lipofectamine 2000. Viruses were harvested 48 hours post-transfection. The Xpress-tagged epsin 1 was previously described[12].

Human Breast Cancer Xenograft Model

Control (Ctrl), epsins 1 and 2 knockdown (KD) or epsin 1 overexpressing (OE)

MDA231 or MCF7 cells were implanted orthotopically into nude mice or subcutaneously into SCID mice as previously described[4]. We recognized tumors more than 2 mm in diameter as positive. Tumor growth was monitored by measuring tumor size using a digital caliper. Tumor volumes were calculated based on the formula: 0.5326 (length [mm] x width^2 [mm]^2). Tumors were harvested, weighed, photographed, embedded and processed for staining as described below.

Histological Examination

Human tissue microarray test slides of 18 cases of normal, reactive and neoplastic conditions of the breast were obtained from BioChain. Mouse tissues were harvested, fixed in 10% formalin and embedded in paraffin. All sections were stained by immunohistochemistry (IHC) or immunofluorescence (IF) as described below.

Immunohistochemistry and Immunofluorescence

For IHC staining, sections were deparaffinized with xylene and alcohol series. After inactivation of endogenous peroxidase with 0.3% hydrogen peroxide and blocking with normal blocking serum, the sections were incubated with corresponding primary antibodies overnight at 4°C. After washing, the sections were incubated with biotinylated secondary antibody in the Vectastain Elite ABC staining kit (Vector Laboratories) for 1 h at room temperature, followed by treatment with the ABC reagent for 30 min. Aminoethyl carbazole (AEC) was used as the peroxidase substrate. Sections were then washed, counterstained with hematoxylin, mounted and photomicrographs obtained. Tissues stained with omission of primary antibody were captured using the same settings and used as negative controls.

For IF staining, sections were blocked as described above and then incubated with the specified primary antibodies overnight at 4°C, followed by incubation with the respective secondary antibodies conjugated to fluorescent labels (Alexa Flour 594 or 488) (Invitrogen) for 1 h at room temperature. Slides were washed, stained with DAPI, mounted and photomicrographs obtained as previously described [4]. Tissues stained with omission of primary antibody were captured using the same settings and

used as negative controls.

Whole Mount Staining of Mammary Glands

Fourth (inguinal) mammary glands were excised, spread onto glass slides, fixed in Carnoy's fixative for 2-4 h at room temperature and then washed in 70% ethyl alcohol for 15 min. Samples were rehydrated by gradually changing the alcohol to distilled water. Once hydrated, the mammary squashes were stained overnight in 0.2% carmine alum and 0.5% aluminum sulfate. The samples were then dehydrated using stepwise ethanol concentrations and left in xylene to clear the fat. Mammary squashes were stored in methyl salicylate. Whole mounts were digitally photographed with a ruler on a stereomicroscope, using the same magnification and lighting conditions. Total area measurements of tumor foci were quantified using NIH Image J software.

Electrophoretic Mobility Shift Assay (EMSA)

We followed the user manual of Nuclear Extraction Kit (Signosis, Inc) for subcellular fractionation. Briefly, the cells were washed and sampled in 500 µL of cold PBS. Pelleted cells (2×10^6) were resuspended in hypotonic buffer (Buffer I) and rocked on ice at 200 rpm for 10 min on a shaking platform. After centrifuge at 12,000 rpm for 5 min at 4°C, the supernatant was discarded thoroughly and completely, and the pellet, representing the nuclear fraction, was washed with Buffer II and rocked on ice at 200 rpm for 2 h on a shaking platform. After centrifuge at 12,000 rpm for 5 min at 4°C, the supernatant, representing the nuclear extract, was transferred to a new tube and subjected to the following procedure, in which we followed the user manual of NF-κB EMSA Kit (Signosis, Inc). The nuclear extract was incubated with biotin-labeled (hot) or unlabeled (cold) NF-κB probes, in the presence or absence of NF-κB antibody (1:100 dilution), at 22°C for 30 min in PCR machine. And then the reaction complex was separated on a non-denaturing polyacrylamide gel. The gel was transferred to a nylon membrane and detected using Streptavidin-HRP conjugate and a chemiluminescent substrate. The shifted bands corresponding to a complex of the NF-κB transcription factors and the labeled NF-κB probes were visualized in comparison to the unbound probes.

RNA Isolation and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted using Trizol Reagent. One microgram of total RNA was treated with 1U RNase-free DNase I to eliminate genomic DNA. The first strand cDNA was synthesized using the SuperScript III First-Strand Synthesis SuperMix. An aliquot of 1 μL of the product was subjected to qRT-PCR in a 7300 Real-Time PCR System (Applied Biosystems) using SYBR Green PCR Master Mix reagent as the detector. PCR amplification was performed in triplicate on 96-well optical reaction plates and replicated in three independent experiments. Gel electrophoresis and melting curve analyses were performed to confirm correct PCR product sizes and absence of nonspecific bands. All qRT-PCR primer sequences are available upon request.

Microarray Analysis

RNA was collected from control and KD MDA231 cells using the RNAeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Gene expression profiles of the MDA231 cells with and without epsins 1 and 2 expression were determined using Agilent human GE 4x44k microarrays (Agilent, G4112F) following the manufacturer's instructions (G4140-90051). RNA samples and universal human reference RNA (Strategene) were labelled with CTP-cy5 and CTP-cy3, respectively. The expression value of individual probes refers to the Log2(Cy5/Cy3) ratio.

Accession Numbers

The raw and normalized microarray data have been deposited in the Gene Expression Ominbus (GEO) database under accession number GSE63828. http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=qhsjocmadlebpoh&acc=GSE63 82 8

Gene Set Enrichment Analysis (GSEA)

Gene set enrichment analysis (GSEA) v2.0 was used to perform the GSEA on various functional and/or characteristic gene signatures as described previously[13]. Normalized microarray expression data were rank-ordered by differential expression between cell populations and/or genetic background as indicated, using the provided

ratio of classes (that is, fold change) metric. Statistical significance was assessed by comparing the enrichment score to enrichment results generated from 1,000 random permutations of the gene set to obtain P values (nominal P value).

Immunoprecipitation and Western Blotting

For immunoprecipitation, transfected HEK 293T cells were lysed with RIPA buffer (1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholic acid, 5 mM tetrasodium pyrophosphate, 50 mM sodium fluoride, 5 mM EDTA, 150 mM NaCl, 25 mM Tris, pH 7.5, 5 mM Na_3VO_4, 5 mM N-ethylmaleimide and protease inhibitor cocktail). For NEMO ubiquitination experiments, cells were lysed using one volume of denaturing buffer (1% SDS in 50 mM Tris, pH 7.5) and boiled for 10 min at 100°C to denature protein complexes. Lysates were re-natured using nine volumes of ice-cold RIPA buffer then prepared for immunoprecipitation as follows. Cell lysates were pre-cleared with mouse IgG and protein G Sepharose beads at 4°C for 2 h followed by incubation with antibodies against HA- or Xpress-epsin 1 or Flag-NEMO at 4°C for 4 h. For negative controls, mouse IgG was added instead of specific antibodies. Precipitated proteins were eluted from beads using 2% SDS in 50 mM Tris, pH 7.5 and visualized by Western blotting. Proteins were resolved by SDS-PAGE (7.5% acrylamide) followed by electroblotting to a nitrocellulose membrane and blocking with 5% milk (w/v). Primary antibodies were incubated at 4°C overnight, followed by incubation at room temperature with the respective horseradish peroxidase-conjugated secondary antibody for 1 h. The immunoreactive proteins were detected by enhanced chemiluminescence with autoradiography. Western blots were quantified using NIH Image J software.
MDA231 cells were cultured in 10% FBS DMEM medium in 10 cm dishes and cells were split and cultured overnight. Forward transfection siRNA for epsin 1 and 2 were performed using RNAiMAX (Invitrogen) according to manufacturer's instruction. After 3 days, cells were detached and reverse transfected in the 2nd round of siRNA for another 3 days. No antibiotics were present in the medium when transfection was conducted. Cells were stimulated by 50 ng/ml TNFα in 0, 5, 15, and 30 minutes. Cells were then washed with 1x PBS for 2 times and lysed in the lysis buffer containing 20 mM Tris-HCl, 137 mM NaCl, 2 mM EDTA, 10% Glycerol, 1% Triton-X100, 0.5%

sodium deoxycholate, 1x cocktail protease inhibitor (Roche), and 50 mM NEM (N-Ethylmaleimide). Anti-Epsin 1 antibody (10 µl, goat anti-human, Santa Cruz Biotechnology Inc) and 35 µl of A/G beads (Santa Cruz Biotechnology Inc) were added into cell lysates and rotated at 4°C overnight. Beads were washed with IP buffer for 2 times, followed by ½ IP buffer (diluted by 1x PBS) for another 2 times. Beads were then suspended in 60 µl 2x loading buffer and heated at 95°C for 2x5 minutes. Beads were Spun down, and supernatant were loaded to SDS-PAGE gel for electrophoresis and transferred onto NC membranes. NC membranes were blotted with Epsin-1, HOIP, HOIL-1L, NEMO, and β-actin antibodies as indicated in the blots.

Pull-down Assays

For GST-NEMO protein fragment pull-downs of HA-tagged epsin 1 full length or truncation mutants, human NEMO fragment (residues G257-S346) was first cloned into the EcoRI and NotI restriction sites of pGEM-T vector (Promega) and then cloned into pGEX-6p-1 vector (GST-tag at N-terminus and 6x His-tag at C-terminus). pGEX-6p-1- NEMO was transformed into BL21 and production of NEMO protein was induced with IPTG at 20°C for overnight. GST fusion proteins were purified either with Glutathione Sepharose 4B (GE Healthcare) or Ni-NTA beads according to the manufacturer's instructions. Proteins were further purified by size-exclusion chromatography (HiLoad 16/600 Superdex 75 column, GE Healthcare Life Sciences) in the buffer containing 20 mM Tris, 150 mM NaCl, pH 8.0. HEK 293T cells were transfected with HA-tagged epsin 1, ENTH, or DPW-NPF constructs described previously[2, 12]. 24 hours after transfection, cells were lysed in the buffer containing 30 mM Tris-HCl (pH 7.4), 120 mM NaCl, 2 mM EDTA, 2 mM KCl, 1% Triton X-100, 5 mM NEM, 4 mM Na_3VO_4 and 1x complete protease inhibitor. Lysates were cleared by centrifugation at 13,200 rpm for 20 min at 4°C before incubation with GSH agarose beads with or without GST-NEMO in binding buffer containing 30 mM Tris-HCl (pH 7.4), 120 mM NaCl, 2 mM EDTA, 2 mM KCl, 1% Triton X-100. Cells lysates containing equal amounts of HA-tagged Epsin 1, ENTH, or DPW-NPF were added, respectively, to the mixtures of either GSH beads or GST- fusion-NEMO beads and were incubated overnight at 4°C on a rocker. The beads were then washed three

times with the binding buffer, resuspended in the Laemmli buffer and boiled, and the supernatant was analyzed by immunoblotting.

For biotinylated UIM pull-downs of NEMO from human breast cancer cells, UIM and the control scrambled peptide were biotinylated as previously described[14], and 100 μg of which were added to 0.5 mL MDA231 cell lysates (1 mg total protein) in the binding buffer containing 30 mM Tris-HCl (pH 7.4), 120 mM NaCl, 2 mM EDTA, 2 mM KCl, 0.05% NP-40, 10% Glycerol, 5 mM NEM, 4 mM Na_3VO_4 and 1x complete protease inhibitor. 40 μL NeutrAvidin Agarose Resin (Thermo fishier Scientific) was added into the solution and incubated overnight at 4°C on a rocker. The resin beads were then washed three times with the binding buffer, resuspended in the Laemmli buffer and boiled, and the supernatant was analyzed by immunoblotting.

For biotinylated UIM pull-downs of linear Ub, K48- or K63-linked Ub, Neutravidin agarose resin containing equivalent amounts of UIM were mixed with non- hydrolyzable tetramer linear Ub, K48-linked Ub, and 63-linked Ub (BostonBiochem), respectively, in the binding buffer containing 30 mM Tris-HCl (pH 7.4), 120 mM NaCl, 2 mM EDTA, 2 mM KCl, 1% Triton X-100. The mixtures were incubated overnight at 4°C on a rocker. The resin beads were then washed three times with the binding buffer, resuspended in the Laemmli buffer and boiled, and the supernatant was analyzed by immunoblotting.

Confocal Imaging of Cells

Cells were fixed in 4% paraformaldehyde and permeabilized in 0.2% Triton X-100/0.02 M PBS, followed by blocking for 1 hour with normal goat serum blocking solution. Cells were incubated with goat anti-epsin 1 or rabbit anti-p65 overnight at 4°C and then incubated with Alexa Fluo 594 nm-conjugated donkey anti-goat and/or Alexa Fluo 488 nm-conjugated donkey anti-rabbit secondary antibodies for 45 min at room temperature in the dark. Cells were then washed, coverslips mounted, and photomicrographs obtained using an Olympus IX81 Spinning Disc Confocal Microscope with an Olympus plan Apo Chromat 60x objective and Hamamatsu Orca-

R2 Monochrome Digital Camera. Colocalization of p65 in the nucleus was quantified in 30 cells.

Cell Surface Biotinylation of TNFR1

Control and epsin-deficient cells were serum starved overnight and then stimulated with TNFa for indicated times. Cells were placed on ice and incubated with 1 nM EZ-Link Sulfo-NHS-LC-Biotin dissolved in cold PBS at 4°C for 30 min with gentle rocking and then washed with cold PBS/50 mM glycine to stop biotinylation. Cells were lysed in RIPA buffer and processed for streptavidin bead pull down. Pull downs from prepared lysates were separated by SDS-PAGE and analyzed by Western blotting. Biotinylated Cell Surface TNFR1 was visualized by Western blotting using anti-TNFR1.

Peptide Synthesis

UIM Peptide was synthesized by FMOC solid phase. The crude product was dissolved, separated and confirmed by mass spectrometry as described in the Online-only Extended Experimental Procedures. After confirmation, peptides were purified by HPLC and lyophilized. Peptide endotoxin (EU/mg) was monitored using LAL kit (Pierce) per manufacturer's instructions. The peptide sequences are available upon request.

Patient-derived Xenografts

NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wj}l/SzJ (NSG, Stock No: 005557) female mice were purchased from The Jackson Laboratory and maintained in standard conditions. At 4 weeks of age, fragments of the triple-negative breast cancer patient-derived xenograft (PDX, HCI-019) were orthotopically implanted into mice as previously described[15]. Mice were anesthetized with isofluorane by being placed in an induction chamber and then transferred to a surgical platform. Continued isofluorane was administered during the procedure via a nose cone. The surgical site is washed with 70% ethanol and shaved. The site was then prepared by alternating alcohol and betadine scrub two times starting with betadine scrub. 1-3 minutes prior to the

procedure, a local anesthetic of 5 mg/kg of Lidocaine was be administered subcutaneously at the presumptive site of incision.

Breast PDX, HCI-019 (manuscript in preparation), tumor fragments (~1-3 mm^3) were transplanted by direct insertion into the remaining fat pad. The incisions were closed with wound clips and removed 7-10 days after surgery. Mice were allowed to recover on a 37 ^oC circulating warm water pad before being returned to a cage. Tumors were measured every 3 days (volume = (width x width x length)/2). When tumors reached 150-250 mm^3, animals were treated with vehicle (PBS) or 40 mg/kg of UIM peptide via intravenous tail vein injections. Animals were dosed three times per week and tumors were harvested when reaching 20 mm on the largest dimension.

Statistical Analysis

Data were shown as mean ± SD if not otherwise indicated. Data were analyzed by the two-tailed Student's t test or ANOVA, where appropriate. The Wilcoxon signed-rank test was used to compare data that did not satisfy the Student's *t* test or ANOVA. p value ≤ 0.05 was considered significant.

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