Supplementary Figure S1

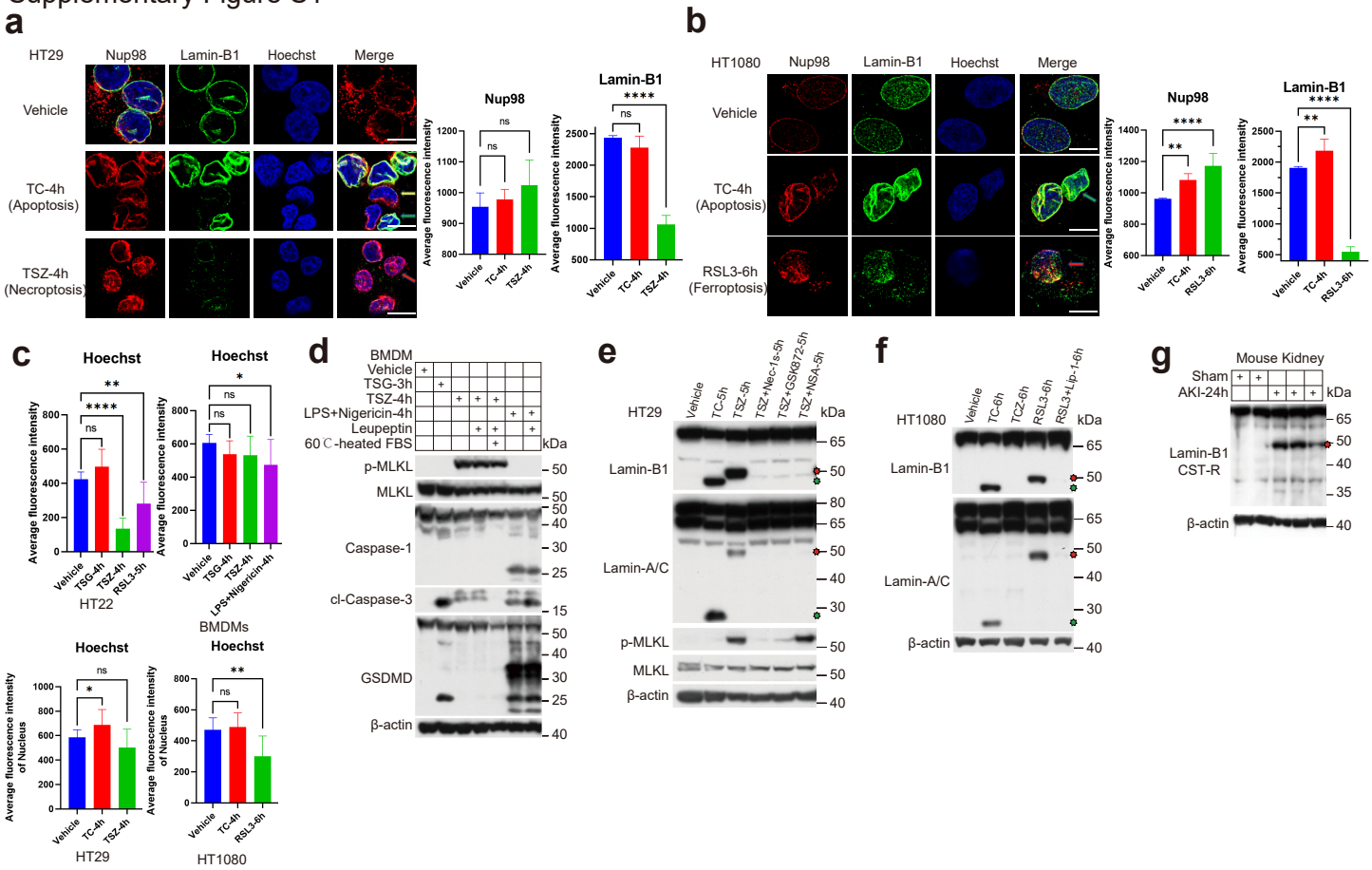


Fig. S1 Destruction of nuclear envelope and lamin protein cleavages in necrotic cells

a, **b** Immunofluorescence analysis and quantification of Nup98 and lamin-B1 in HT29 cells (**a**) and HT1080 cells (**b**) treated with indicated compounds for indicated time. Arrows indicate morphological phenotypes: red arrows, necrotic morphology; green arrows, apoptotic morphology; yellow arrows, secondary necrosis. Statistical analyses were performed on 10 fields with at least 50 cells per condition. Data are shown as mean ± SD (*n* = 10 fields). Statistical significance was determined using one-way ANOVA with Sidak's multiple comparison test. Scale bars, 10 μm. Unless otherwise indicated, details of compound treatment and concentrations are described in the Methods section.

c Quantification of Hoechst signals from Fig. 1c, d and Supplementary Fig. S1a, b. Statistical analyses were performed on 10 fields with at least 50 cells per condition. Data are shown as mean ± SD (*n* = 10 fields). Statistical significance was determined using one-way ANOVA with Sidak's multiple comparison test.

d–**f** Western blotting analysis of BMDMs (**d**), HT29 cells (**e**) and HT1080 cells (**f**) treated with indicated compounds for indicated time. Red star marks necrotic cleavage products, and green star marks apoptotic cleavage products. The BMDMs lysates were also used for western blotting in Fig. 3g. Unless otherwise noted, each western blot panel is from a single experiment. The overall conclusions are supported by independent experiments performed under comparable conditions.

g Western blotting analysis of kidney tissues from 8-week-old male wild-type mice 24 h after sham operation or unilateral ischemia-reperfusion acute kidney injury (AKI, 70 min ischemia). Lamin-B1 CST-R indicates the rabbit anti-lamin-B1 antibody from CST; unless otherwise indicated, the lamin-B1 antibody used was the mouse antibody from Proteintech. Red star marks necrotic cleavage products.

Supplementary Figure S2

a

Cocktail protease inhibitor

Inhibitor	Concentration	Target
AEBSF	125 μM	Serine proteases
Aprotinin	250 nM	Serine proteases
Bestatin	50 μM	Aminopeptidases
E-64	10 μM	Cysteine proteases
Leupeptin	50 μM	Serine and cysteine proteases
Pepstatin A	25 μM	Aspartic proteases
TLCK	50 μM	Serine proteases
Cathepsin inhibitor 1	200 μM	Cathepsin L/2/S/K/B
Z-FY-CHO	200 μM	Cathepsin L
LY2811376	160 μM	Cathepsin D/BACE1
Calpeptin	500 μM	Calpain
FMK-9a	150 μM	ATG4B
NH_4Cl	10 mM	Lysosome
Chloroquine	75 μM	Autophagy and Toll-like receptors
Bafilomycin A1	1 μM	V-ATPase
E64d	200 μM	Cysteine protease
Bardoxolone-Me	600 μM	Lon protease
Ilomastat	150 μM	Matrix metalloprotease
MG132	20 μM	Proteasome
Suc-AAPF-CMK	108 μM	Unknown target

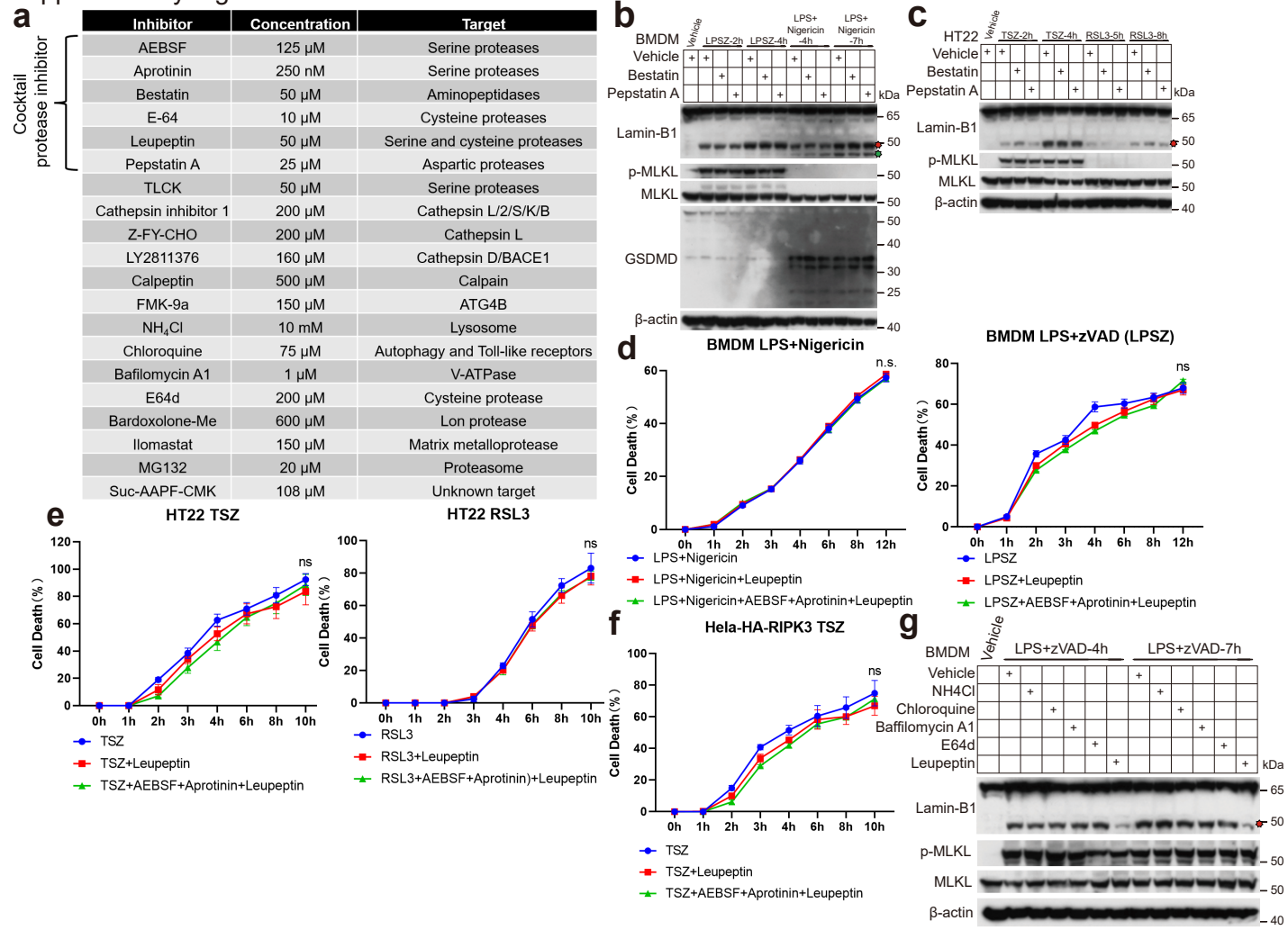


Fig. S2 Bestatin, Pepstatin A and lysosomal protease inhibitors do not affect necrotic cleavage of lamins

a List of protease inhibitors, concentrations used and their targets. This figure is related to Fig. 2a.

b, **c** Western blotting analysis of BMDMs (**b**) and HT22 cells (**c**) treated with indicated compounds for indicated time. AEBSF (125 μM), Aprotinin (250 nM), serine and cysteine protease inhibitor leupeptin (50 μM), cysteine protease inhibitor E64 (10 μM), aminopeptidase inhibitor Bestatin (50 μM), or aspartic proteases inhibitor Pepstatin A (25 μM) were applied as indicated. Red star marks necrotic cleavage products, and green star marks apoptotic cleavage products.

d–**f** Quantification of cell death by SYTOX Green. BMDMs (**d**), HT22 cells (**e**) and HeLa cells expressing HA-RIPK3 (HeLa-RIPK3, **f**) were treated with indicated compounds. Leupeptin, 50 μM; AEBSF, 125 μM; Aprotinin, 250 nM. Data are shown as mean ± SD (n = 3 biological replicates). Statistical significance was determined using two-way ANOVA with Dunnett's multiple comparison test. ns indicates the comparison at the time point of 10 h or 12h shown in the figure. This figure is related to Fig. 2b-d.

g Western blotting analysis of BMDMs treated with indicated compounds for indicated time. NH4Cl, 10 mM; Chloroquine, 75 μM; Bafilomycin A1, 1 μM; E64d, 200 μM; Leupeptin, 50 μM. Red star marks necrotic cleavage products.

Supplementary Figure S3

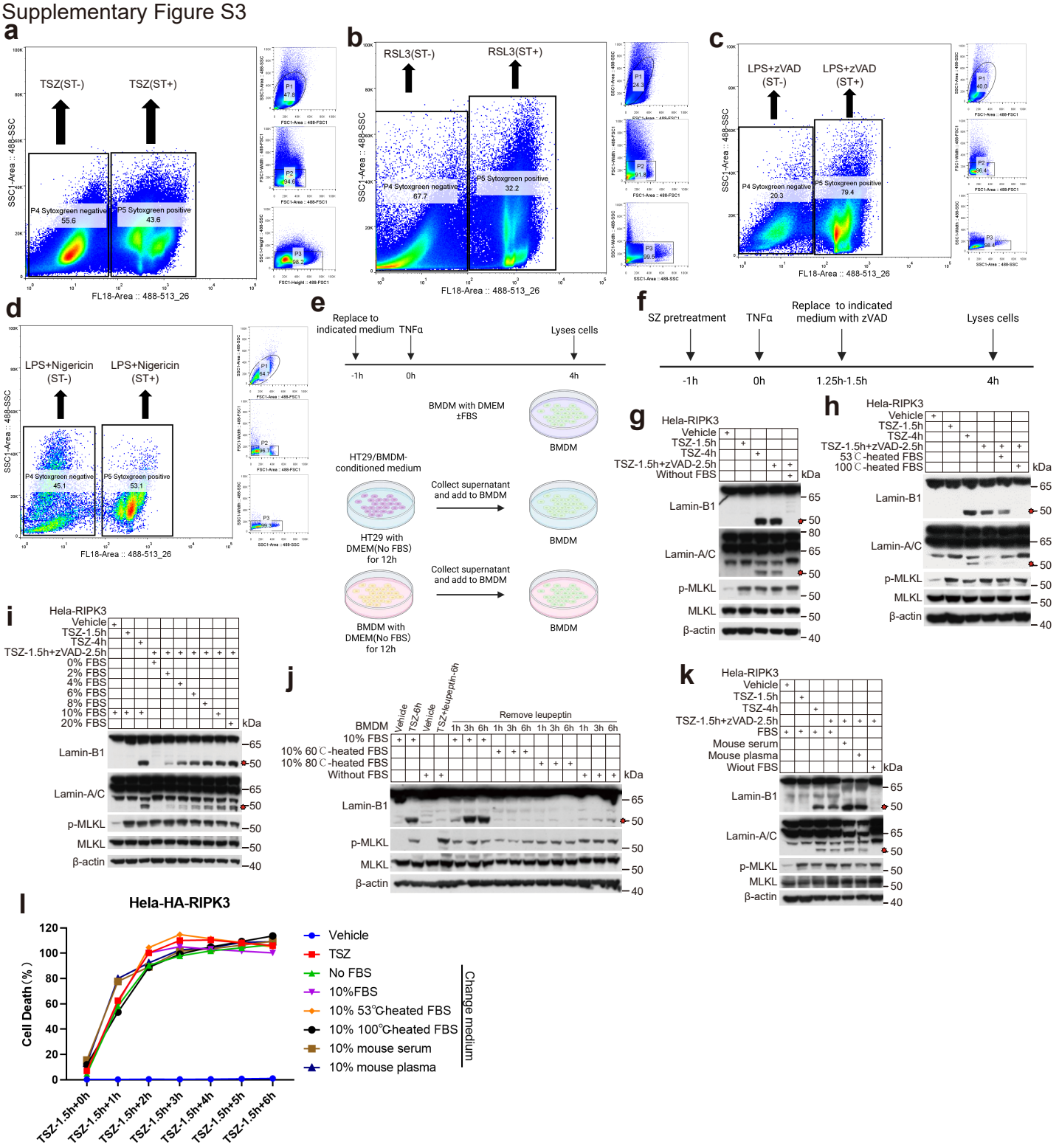


Fig. S3 Extracellular proteases mediate necrotic protein cleavage

a–**d** Flow cytometry analysis of SYTOX Green-positive (ST^+)/negative (ST^-) cells. HT29 cells were treated with TSZ for 4.5 h (**a**); HT22 cells were treated with RSL3 for 5 h (**b**); BMDMs were treated with LPS+zVAD for 3.5 h (**c**), or LPS+Nigericin for 5 h (**d**). The cells were stained with SYTOX Green (1 μM) and analyzed by FACS. P4 indicates SYTOX Green negative population (ST^-), and P5 indicates SYTOX Green positive population (ST^+). This figure is related to Fig. 2g-i.

e Schematic representation of treatments in Fig. 2j. This figure was created by BioRender.com.

f Schematic representation of treatments in Supplementary Fig. S3g-i and k. This figure was created by BioRender.com.

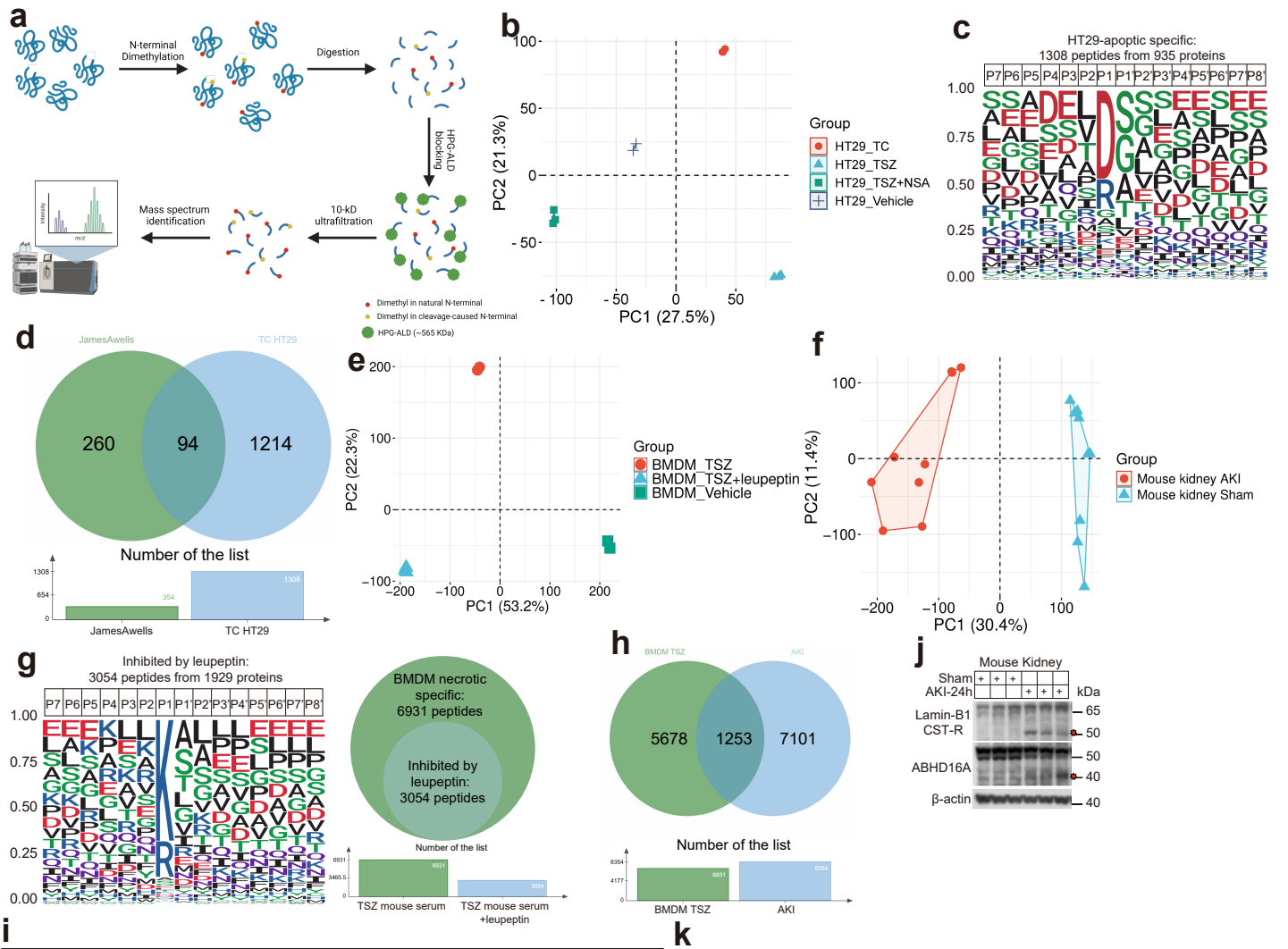
g–**i** Western blotting analysis of HeLa-RIPK3 cells treated with TSZ for 1.5 h and then replaced with indicated medium (**g**); indicated medium with FBS subjected to different heat treatments (**h**); indicated medium containing different concentrations of FBS (**i**), for another 2.5 h. Red star marks necrotic cleavage products.

j Western blotting analysis of BMDMs treated with TSZ+leupeptin (100 μM) in DMEM without FBS for 6 h, followed by incubation in indicated media for 1, 3, or 6 h. Red star marks necrotic cleavage products.

k Western blotting analysis of HeLa-RIPK3 cells treated with TSZ for 1.5 h and then replaced with indicated medium containing mouse serum or mouse plasma for another 2.5 h. Red star marks necrotic cleavage products.

l Cell death measured by SYTOX Green: HeLa-RIPK3 cells were treated with TSZ for 1.5 h and then replaced the medium with indicated medium and SYTOX Green (250 nM). This figure is related to Supplementary Fig. S3g, h and k.

Supplementary Figure S4



i

Protein	Cleavage sites identified by N-terminomic analysis in HT29 cells	Cleavage sites identified by N-terminomic analysis in BMDMs	Cleavage sites identified by N-terminomic analysis in mouse AKI model
TMPO/LAP2β	K303(**33.49**)	K302(**33.26**)	K302(33.26)
ABHD16A/ BAT5	R178(**42.75**)	R178(**42.77**)	R178(**42.77**)
ATP13A1	R917+R611(**33.74**)	K913(100.42)/K223(108.47)	K223(108.47)
PREB	K127(**31.99/13.51**)	K224(24.16)/K140(**30.57/14.89**)	K224(24.16)/K360(39.20)
Syntaxin-18	R165(19.20/19.50)	R165(19.13/19.27)	R131(15.04/23.37)
PELP1	R48(115.26)/R531(63.78)	K499(65.32)	K499(65.32)
Lamin-A/C	R321(36.74)/K457(52.43)	K418(47.90)/R298(34.19)	R196(22.03)/R470(53.93)
Lamin-B1	R226(40.93)	K242(39.29)/R456(52.15)	R72(58.68)/K80(57.83)
Lamin-B2	R16(68.02)/R561(63.28)	R55(60.98)/R116(53.98)	K492(56.04)/R226(41.39)

k

Protein	Cleavage site identified by N-terminomic analysis in HT29 cells	Cleavage site identified by N-terminomic analysis in BMDMs	Cleavage site identified by N-terminomic analysis in mouse AKI model
GLUD1	ND	K397(18.10)	K397(**18.10**)/K503(**11.89**)/K527(**14.64**)
TUBA4A	R373(8.63)/R339(12.33)	K280(18.87)/K164(31.93)/K338(12.49)	K352(10.82)/K40(45.53)/K326(**13.69**)/K164(**31.93**)
CYP4B1	ND	ND	K139(42.92)/R306(**23.50**)
PCCB	R259(30.85)	R91(48.51)	K101(47.50)/K250(31.95)/K491(**52.79**)
ZNRF2	ND	ND	R74(**17.09**)/R72
PON1	ND	R290(32.42/7.16)	R290(32.42/**7.16**)
ACAT1	ND	R102(10.75)	K265(16.45)
ST7	R393(45.06)	ND	R393(45.05)/R392(44.89)

Fig. S4 Identification of necrotic protein cleavages by neo-N-terminomic analysis

a Schematic representation of neo-N-terminomic analysis by mass spectrometry. This figure was created by BioRender.com.

b, **e**, **f** Principal component analysis (PCA) of neo-N-terminomic analysis derived from HT29 cells treated with Vehicle, TC-7 h, TSZ-4.5 h, TSZ+NSA-4.5 h, cultured in DMEM supplemented with 10% FBS (**b**); BMDMs treated with Vehicle, TSZ-4 h, TSZ+leupeptin-4 h, cultured in DMEM containing 4% FBS and 6% mouse serum (**e**); mouse kidney tissue samples of Sham-operated (3 mice), and AKI (24 h post-injury; 4 mice) groups (**f**). Each biological replicate of mouse sample (n = 3 for sham group and n = 4 for AKI group) was analyzed in three technical replicates.

c, **g** Protein cleavage sites identified by neo-N-terminomic analysis. **c** 1,004 apoptotic cleavage peptides from 691 proteins were identified in HT29 cells treated with TC. **g** 3054 necrotic cleavage peptides from 1,929 proteins which were inhibited by leupeptin were identified in BMDMs with treatment of TSZ.

d, **h** Venn diagrams illustrating the overlap of cleaved peptides identified in apoptosis of HT29 cells and in a prior analysis by Mahrus et al. (**d**), and cleaved peptides identified in necroptosis of BMDMs and mouse AKI model (**h**).

i, **k** Summary of necrotic cleavage sites identified by neo-N-terminomic analysis in necroptosis of HT29 cells (**i**), and mouse AKI model (**k**). The cleavage sites are indicated. The bracket after cleavage site shows the predicted cleavage molecular weight and the observed cleavage molecular weight was highlighted by bold-type letter. The cleavage sites identified in the same protein by another two neo-N-terminomic analysis in BMDMs and mouse AKI model were also listed. ND: Not detected.

j Western blotting analysis of kidney tissues from 8-week-old male wild-type mice 24 h after sham operation or unilateral AKI (70 min ischemia). Red star marks necrotic cleavage products. n = 3 per group.

Supplementary Figure S5

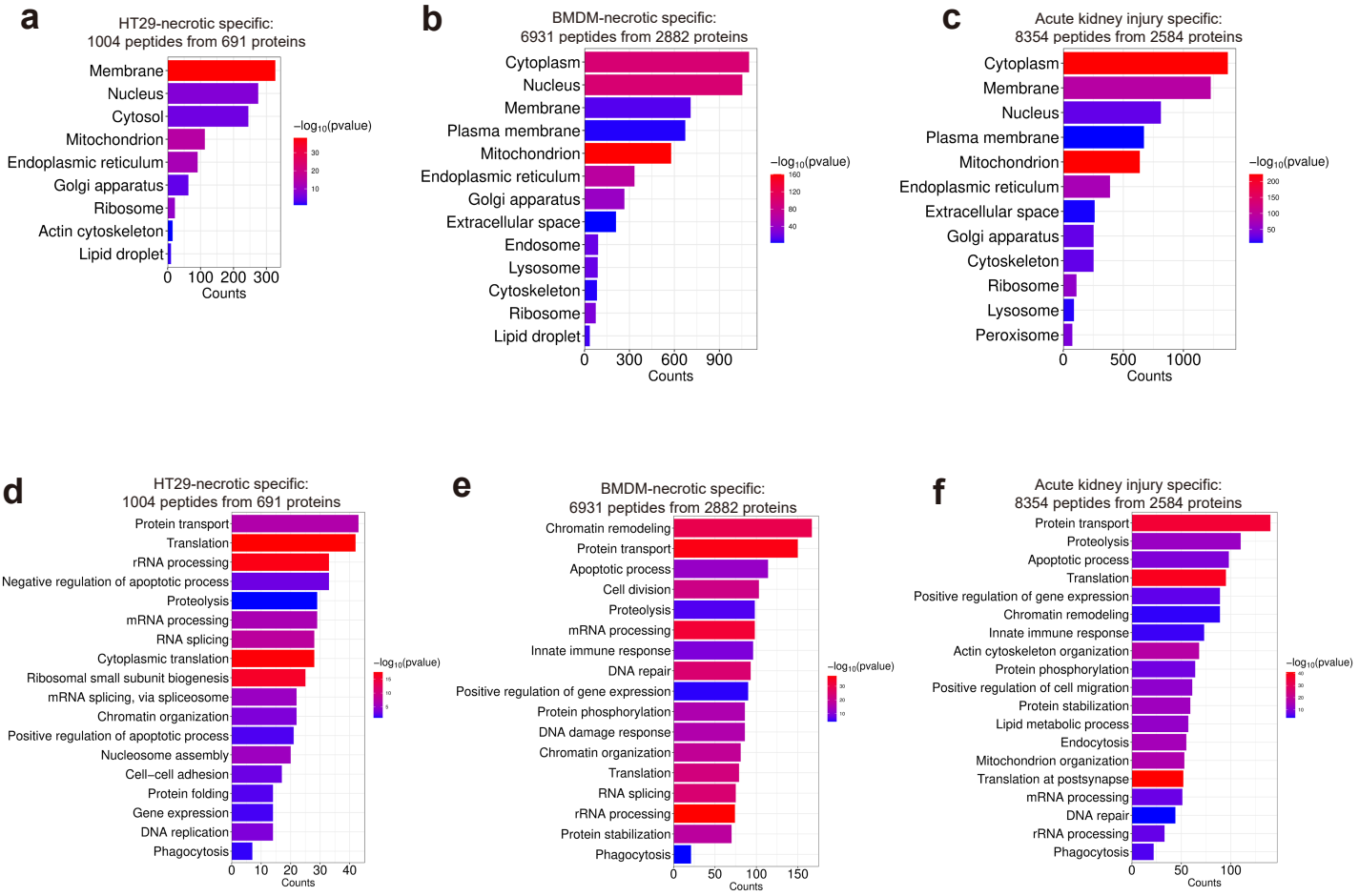
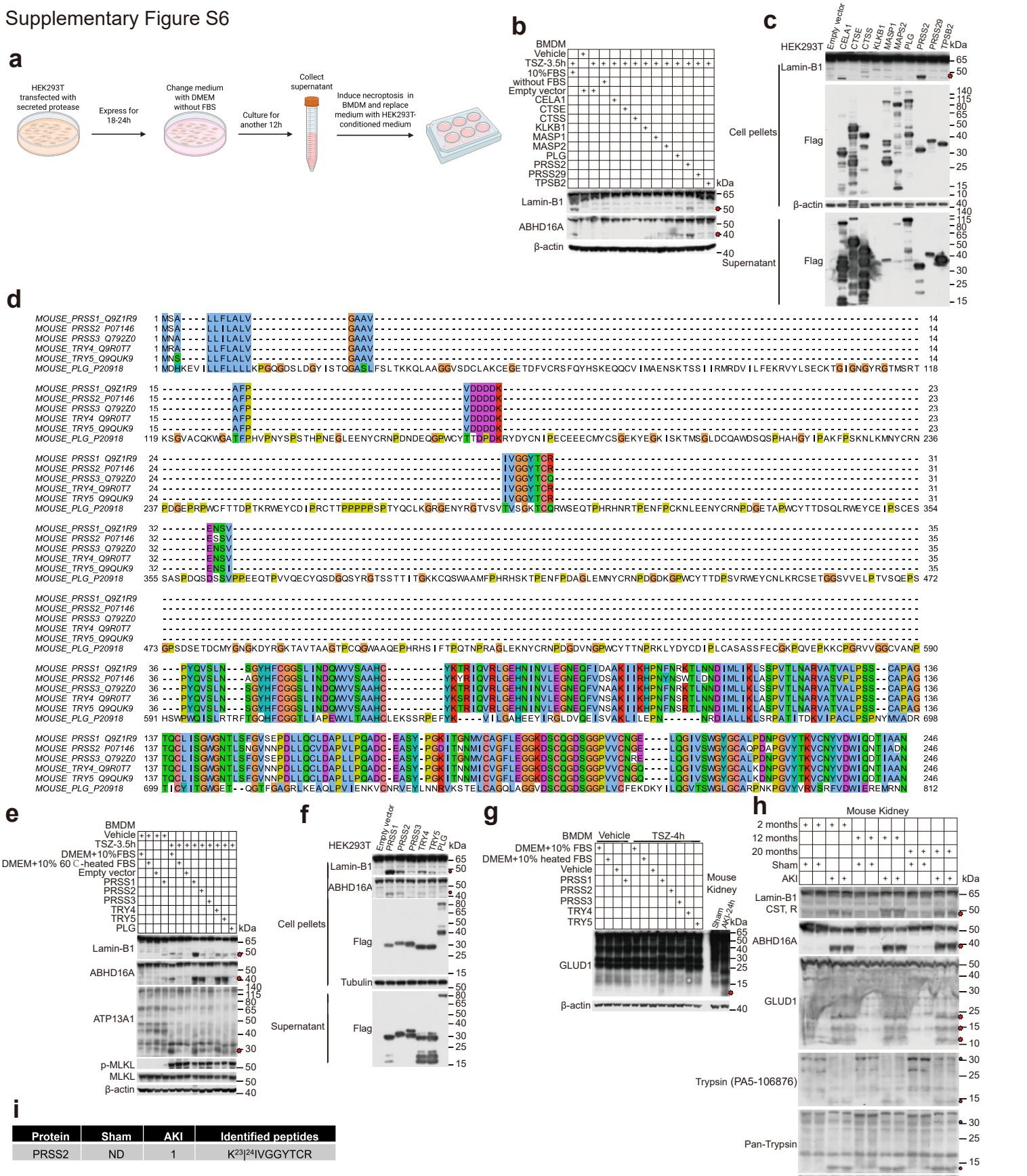


Fig. S5 Cellular component and biological process analysis of cleaved proteins identified in N-terminomic analysis
a–**c** GO cellular component analysis of proteins identified in neo-N-terminomic analysis of HT29 cells (**a**) and BMDMs (**b**) undergoing necroptosis, and mouse kidney tissue samples from the AKI model (**c**). This figure is related to Fig. 3a–c.
d–**f** GO biological process analysis of proteins identified in neo-N-terminomic analysis of HT29 cells (**d**) and BMDMs (**e**) undergoing necroptosis, and mouse kidney tissue samples form the AKI model (**f**). This figure is related to Fig. 3a–c.

Supplementary Figure S6



i

Protein	Sham	AKI	Identified peptides
PRSS2	ND	1	K[23]|[24]IVGGYTCR

Fig. S6 Identification of proteases that can mediate necrotic protein cleavage

a Schematic flow diagram for screening proteases that can mediate necrotic protein cleavage. HEK293T cells were transfected with empty vector or vector expressing proteases. After expressing for 18-24 h, the cultured medium was changed to DMEM without FBS, and then cultured for another 12 h. HEK293T-conditioned medium expressing indicated proteases was collected. This figure was created by BioRender.com. This figure is related to Supplementary Fig. S6b, c and S6e–g.

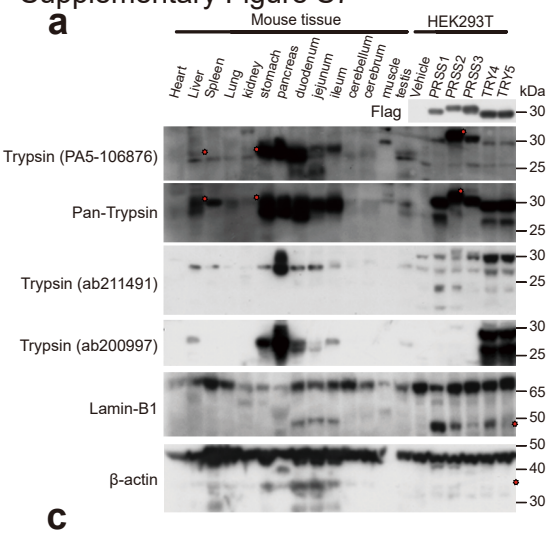
b, **c**, **e**–**g** BMDMs were treated with SM164+zVAD (SZ) for 1 h and then replaced with medium from HEK293T-conditioned medium expressing indicated proteases containing TNFα+SM164+zVAD (TSZ) for another 3.5 h (**b**, **e**) or 4 h (**g**). Mouse AKI model samples from the experiment described in Fig. 3h were included as controls. **c**, **f** HEK293T cells expressing indicated proteases were separated to cells (input) and supernatant. The supernatant was concentrated approximately tenfold using a 10-kDa ultrafiltration device. Cells and supernatant were lysed with 4% SDS and subjected to western blot analysis. Red star marks necrotic cleavage products.

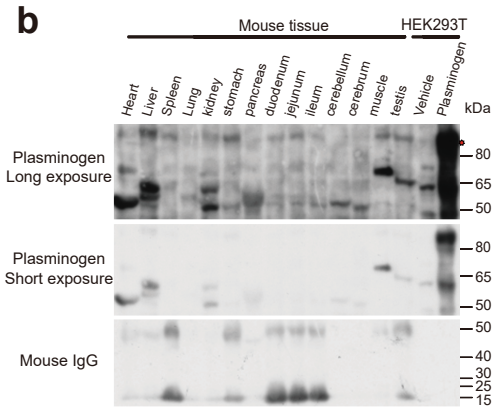
d Amino acid sequence alignment of mouse PRSS1, PRSS2, PRSS3, TRY4, TRY5 and plasminogen by Jalview.

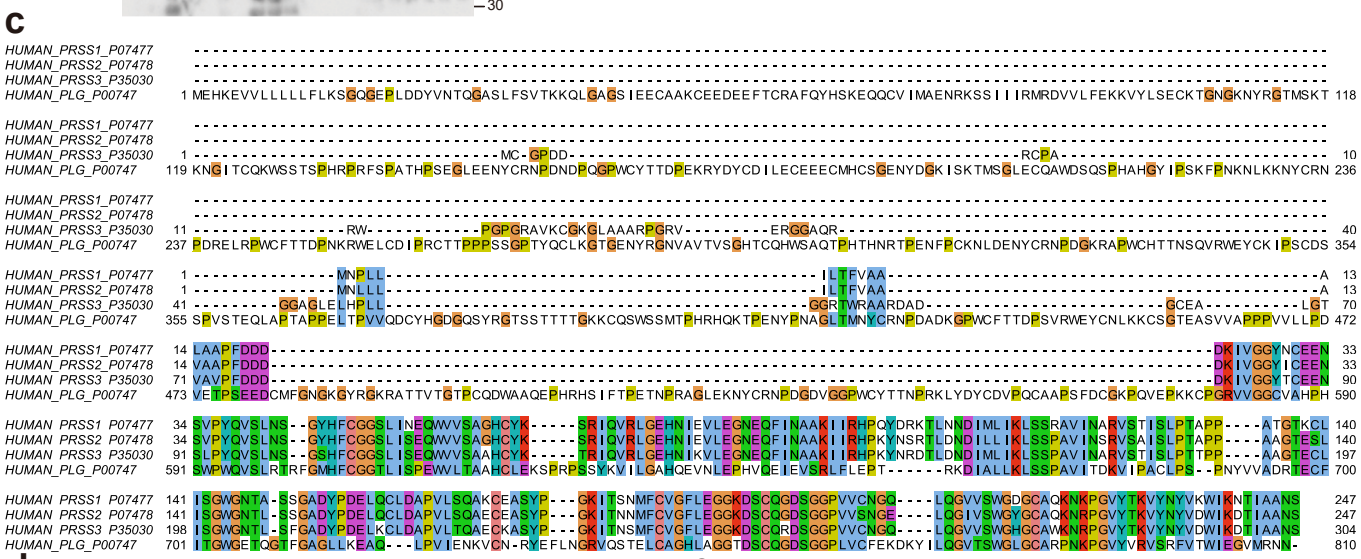
h Western blotting analysis of kidney tissue lysates from 2-month-old, 12-month-old and 20-month-old male wild-type mice 24 h after sham operation or unilateral AKI (70 min ischemia). Red star marks necrotic cleavage products, blue star marks full-length trypsin. *n* = 2 per group.

i Spectral counts of peptide and cleavage site of PRSS2 identified by neo-N-terminomic analysis of mouse AKI model. ND: Not detected.

Supplementary Figure S7







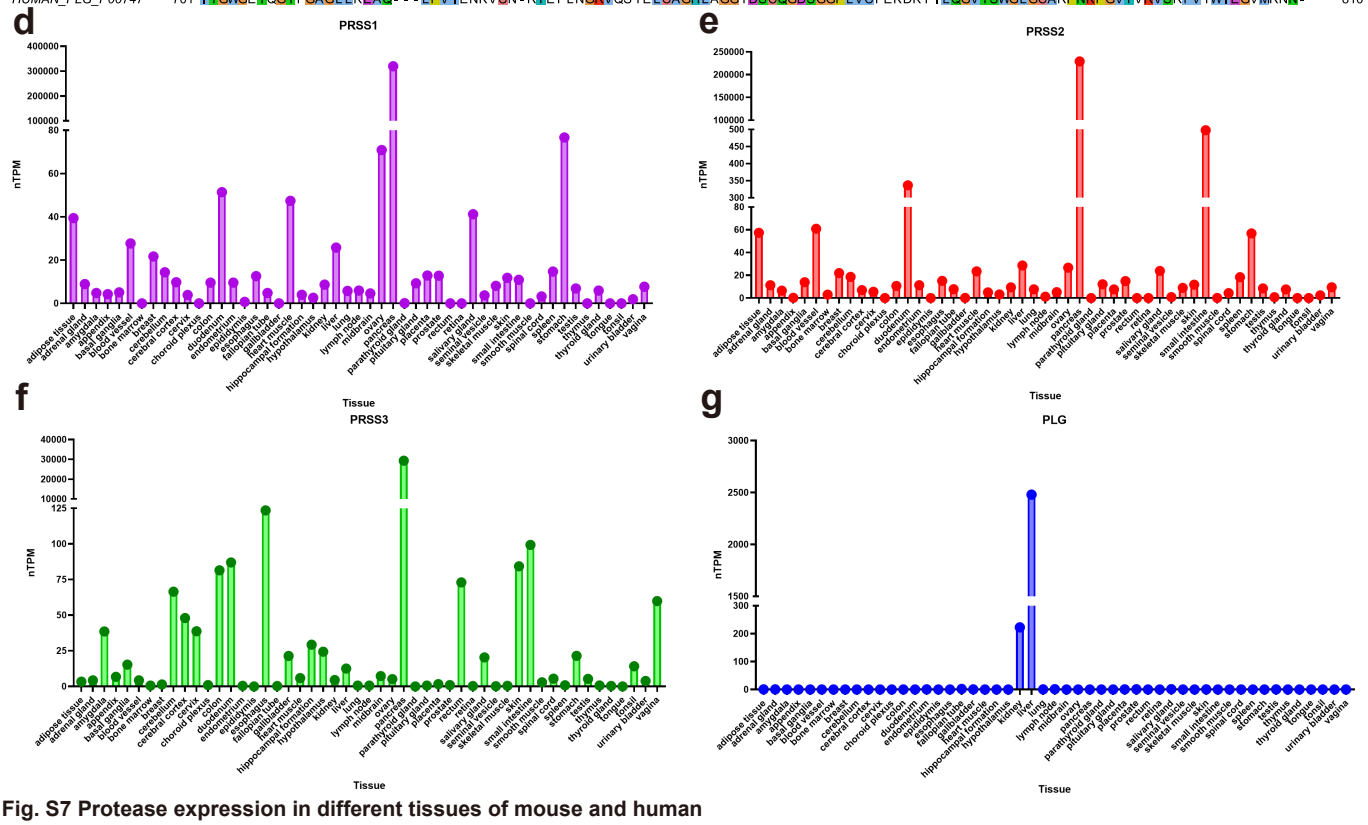


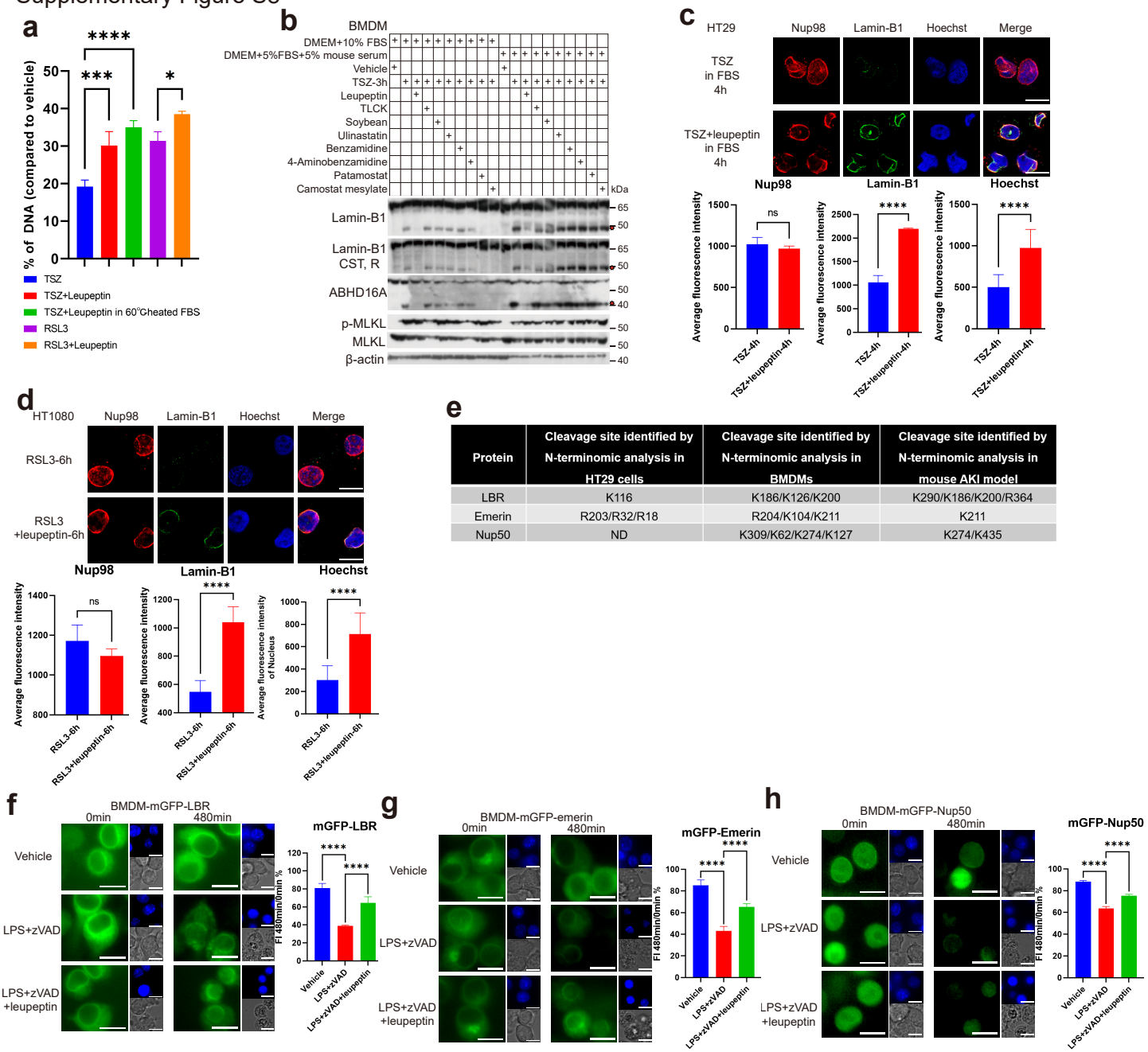
Fig. S7 Protease expression in different tissues of mouse and human

a, **b** Western blotting analysis of indicated mouse tissue lysates (male, aged 8 weeks) and HEK293T cells expressing PRSS1, PRSS2, PRSS3, TRY4, TRY5 (**a**) and plasminogen (**b**). Mouse IgG levels were blotted using an anti-mouse secondary antibody as an indicator of residual blood in perfused mouse tissues, since plasminogen is highly abundant in blood and potential contamination from remaining blood needed to be excluded. The mouse tissue lysates were also used for western blotting in Supplementary Fig. S10c.

c Amino acid sequence alignment of human PRSS1, PRSS2, PRSS3 and PLG by Jalview.

d-g RNA expression levels of *PRSS1*, *PRSS2*, *PRSS3* and *PLG* across human tissues. Data were obtained from the Human Protein Atlas (HPA) using the Consensus dataset integrating HPA and GTEx transcriptomic data. Panels show the tissue RNA expression profiles of *PRSS1* (**d**), *PRSS2* (**e**), *PRSS3* (**f**) and *PLG* (**g**), respectively.

Supplementary Figure S8



Protein	Cleavage site identified by N-terminomic analysis in HT29 cells	Cleavage site identified by N-terminomic analysis in BMDMs	Cleavage site identified by N-terminomic analysis in mouse AKI model
LBR	K116	K186/K126/K200	K290/K186/K200/R364
Emerin	R203/R32/R18	R204/K104/K211	K211
Nup50	ND	K309/K62/K274/K127	K274/K435

Fig. S8 Morphological changes in necrotic cell is mediated by necrotic protein cleavage

a HT22 cells were treated with indicated compounds and indicated medium. The genomic DNA in cells was extracted by Vazyme FastPure Cell/Tissue DNA isolation Mini Kit in triplicate for each data point. Data are shown as mean ± SD (*n* = 3 biological replicates). Statistical significance was determined using one-way ANOVA with Sidak's multiple comparison test.

b Western blotting analysis of BMDMs treated with different trypsin-like protease inhibitors in DMEM supplemented with 10% FBS or DMEM supplemented with 5% FBS and 5% mouse serum. Leupeptin, 50 μM; TLCK, 225 μM; Soybean, 2 mg/mL; Ulinastatin, 0.59 mg/mL; Benzamidine, 125 μM; 4-Aminobenzamidine, 200 μM; Patamostat, 50 μM; Camostat mesylate,100 μM. Red star marks necrotic cleavage products.

c, **d** Immunofluorescence analysis and quantification of Nup98, Lamin-B1 and Hoechst signals in HT29 cells treated with TSZ in the presence or absence of leupeptin (**c**), or in HT1080 cells treated with RSL3 in the presence or absence of leupeptin for indicated time (**d**). Statistical analyses were performed on 10 fields with at least 50 cells per condition. Data are shown as mean ± SD (*n* = 10 fields). Statistical significance was determined using a two-tailed unpaired t-test. Scale bars, 10 μm.

e Cleavage sites of selected nuclear proteins identified in neo-N-terminomic analysis. ND: Not detected.

f–**h** BMDMs expressing pMSCV-mGFP-LBR (**f**), pMSCV-mGFP-emerin (**g**), or pMSCV-mGFP-Nup50 (**h**) were treated with LPS+zVAD in the presence or absence of leupeptin, and were analyzed by HCS. Statistical analyses were performed on 10 fields with at least 500 cells per condition. Data are shown as mean ± SD (*n* = 10 fields). Statistical significance was determined using one-way ANOVA with Sidak's multiple comparison test. Leupeptin, 100 μM. Scale bars, 10 μm.

Supplementary Figure S9

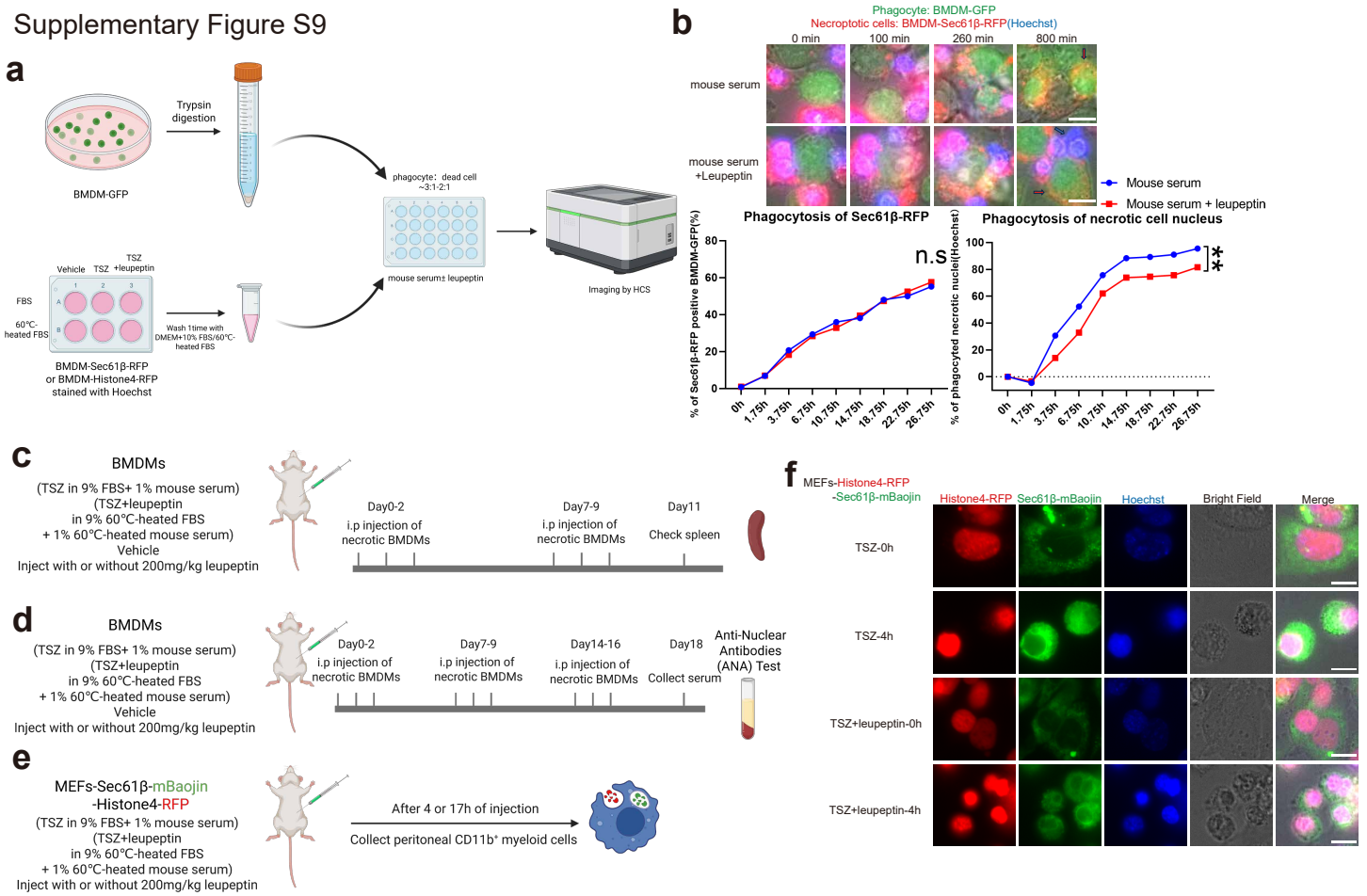


Fig. S9 Necrotic protein cleavage promotes phagocytosis of necrotic nuclei

a Schematic representation of phagocytosis assay in vitro. This figure was created by BioRender.com.

b BMDMs expressing GFP were co-cultured with BMDMs expressing Sec61β-RFP, which were stained with Hoechst (1 μg/mL) and treated with TSZ in medium containing 10% FBS. The phagocytosis was assayed in DEME containing 4% mouse serum+ 2% FBS (mouse serum) or 4% mouse serum+ 2% FBS+leupeptin (mouse serum+leupeptin) and imaged by HCS. Statistical analysis of the percentage of Sec61β-RFP positive BMDM-GFP or percentage of the remaining nuclei marked by Hoechst were based on 10 fields with at least 400 cells per condition. Data from 10 fields were pooled for analysis. Statistical significance was determined using a two-tailed paired t-test. Scale bars, 10 μm.

c–**e** Schematic flow diagrams of intraperitoneal injection of necrotic cells and assessed for weights of spleen (**c**), autoimmunity analysis (**d**), or phagocytosis *in vivo* (**e**). This figure was created by BioRender.com. This figure is related to Fig. 5b–e.

f MEFs expressing Histone4-RFP and Sec61β-mBaojin were inoculated in 24-well plates and treated with TSZ in medium containing 9% FBS+1% mouse serum or with TSZ+leupeptin in medium containing 60 ℃-heated serum (9% FBS+1% mouse serum). Hoechst (1 μg/mL) was added. Images at 0 h and 4 h were presented. Scale bars, 10 μm.

Supplementary Figure S10

a

Protein	Cleavage site identified in mouse AKI model	Cleavage site identified in necroptotic BMDMs	Cleavage site identified in necroptotic HT29 cells	Putative molecular weight of cleaved N - terminal fragment	Putative molecular weight of cleaved C - terminal fragment
ABHD16A/ BAT5	R178	R178	R178	20.35 kDa	42.77 kDa
GLUD1	K397/K503/K527	K397/K503	Not Detected	37.83 kDa	18.10 kDa/ 14.64 kDa/11.89 kDa

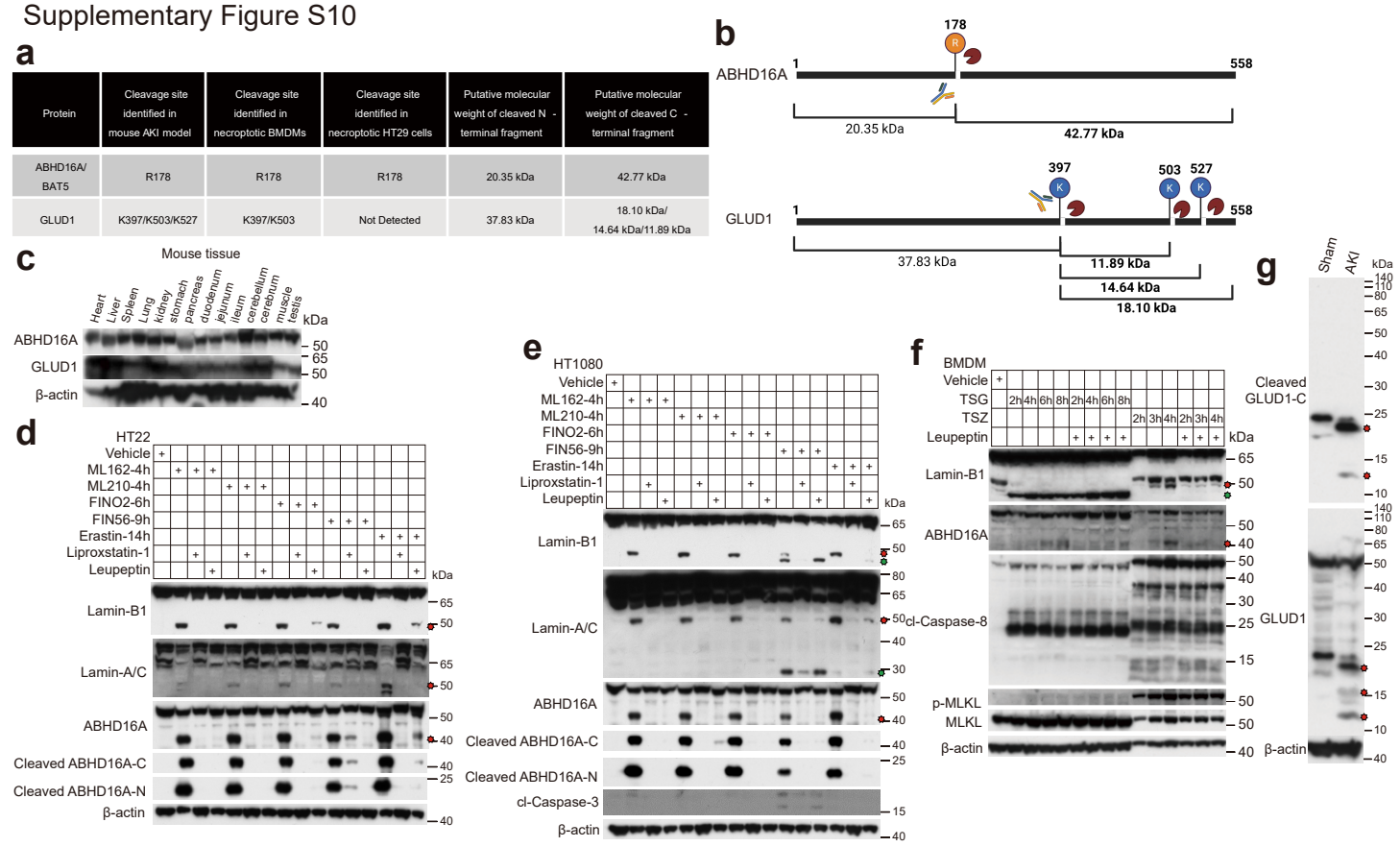


Fig. S10 Validation of necrotic cleavage antibodies by western blotting

a List of cleavage sites and putative molecular weight of cleaved fragments of ABHD16A and GLUD1 identified in neo-N-terminomic analysis by mass spectrometry.

b Schematic representations of the necrotic cleavage sites in ABHD16A and GLUD1, and the putative molecular weight of cleaved fragments. This figure was created by BioRender.com.

c Western blotting analysis of indicated mouse tissue lysates (male, aged 8 weeks). The samples used in Supplementary Fig. S10c and Supplementary Fig. S7a were obtained in the same experiment.

d, **e** Western blotting analysis of HT22 cells (**d**) and HT1080 cells (**e**) treated with ML162 (10 μM), ML210 (10 μM), FINO2 (10 μM), FIN56 (10 μM) and Erastin (10 μM) alone or in combination with Liproxstatin-1 (500 nM) or leupeptin (50 μM) for indicated time. Red star marks necrotic cleavage products. Green star marks apoptotic cleavage products.

f Western blotting analysis of BMDMs treated with TSG or TSZ in the presence or absence of leupeptin for indicated time. Leupeptin, 50 μM. Red star marks necrotic cleavage products, and green star marks apoptotic cleavage products.

g The kidney samples were collected from sham-operated and unilateral AKI mice. Samples were analyzed by western blotting using a monoclonal antibody against cleaved GLUD1-C product. Red star marks necrotic cleavage products.

Supplementary Figure S11

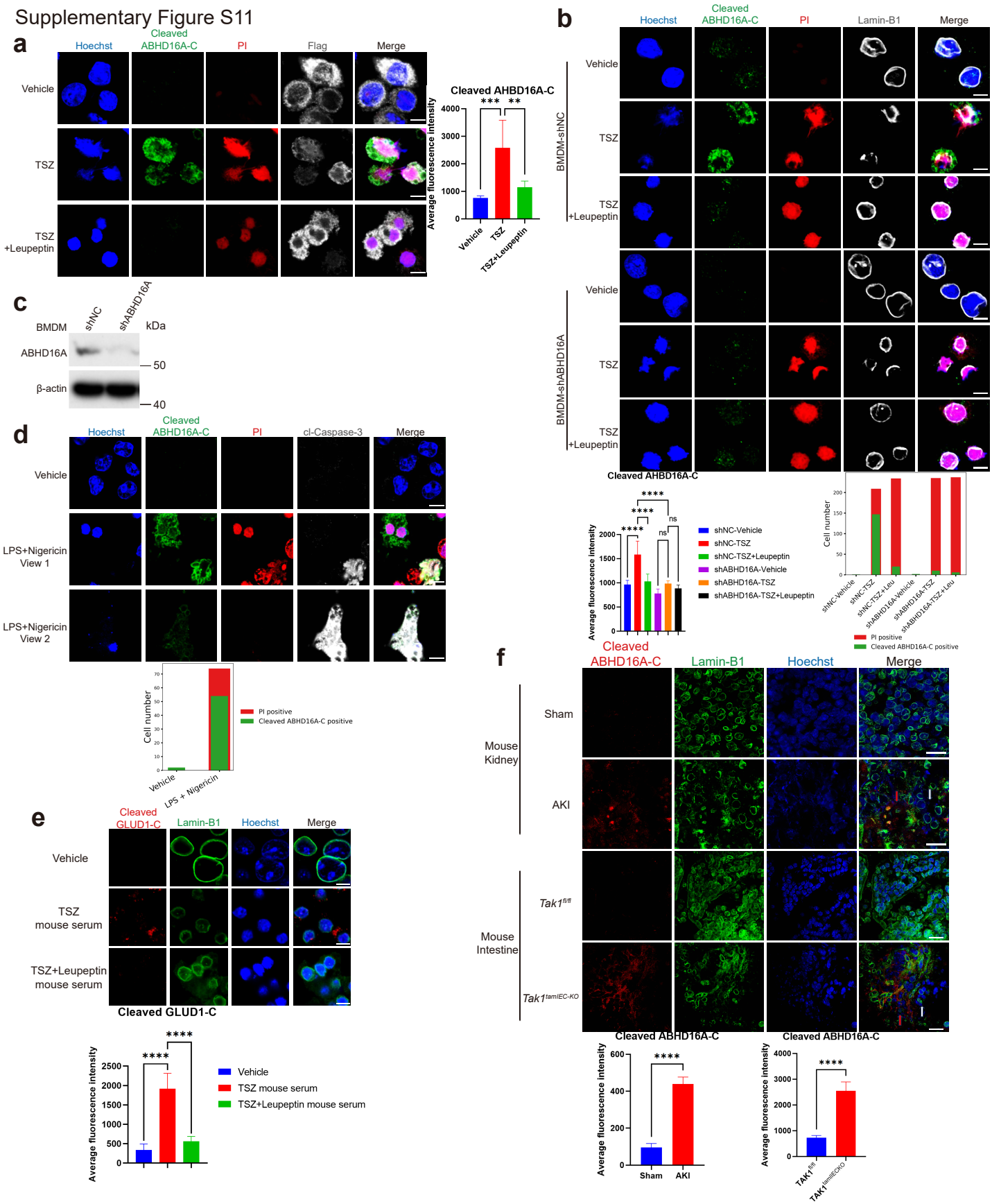


Fig. S11 Validation of necrotic cleavage antibodies by IF

a, **b** BMDMs expressing ABHD16A-3xFlag (**a**), control shRNA (shNC) or shABHD16A targeting ABHD16A for knockdown (**b**) were treated with TSZ in the presence or absence of leupeptin for 3.5 h and then fixed with cold methanol. PI (2 μg/mL) was added 0.5 h before methanol fixation. The cells were co-immunostained using monoclonal antibodies against cleaved ABHD16A-C and Flag (**a**) or cleaved ABHD16A-C and Lamin-B1 (**b**). Statistical analyses were performed on 5 fields with at least 100 cells per condition. Data are shown as mean ± SD (*n* = 5 fields). Statistical significance was determined using one-way ANOVA with Sidak's multiple comparison test. Scale bars, 5 μm.

c Western blotting analysis of cell lysates from BMDMs expressing shNC or shABDH16A.

d BMDMs were treated with LPS+Nigericin for 4 h and then fixed with cold methanol. PI (2 μg/mL) was added 0.5 h before methanol fixation. The cells were co-immunostained for monoclonal antibodies against necrotic ABHD16A-C and cl-Caspase-3. Statistical analyses were performed on 10 fields with at least 200 cells per condition. Scale bars, 5 μm.

e BMDMs were treated with TSZ in the presence or absence of leupeptin in medium containing 4% FBS plus 6% mouse serum for 4 h and then fixed with cold methanol. The cells were co-immunostained for monoclonal antibodies against cleaved GLUD1-C and Lamin-B1. Statistical analyses were performed on 6 fields with at least 100 cells per condition. Data are shown as mean ± SD (*n* = 6 fields). Statistical significance was determined using one-way ANOVA with Sidak's multiple comparison test. Scale bars, 5 μm.

f Mice at 8 weeks of age were subjected to sham operation or unilateral kidney AKI (70 min ischemia) and analyzed after 24 h. *Tak1*$^{fl/fl}$ mice and *Tak1*$^{tamIEC\text{-}KO}$ mice were induced by intraperitoneal injection of tamoxifen (80 mg/kg) for 3 days to induce TAK1 deletion and were sacrificed on day 4. The sections of mouse kidney and small intestine were fixed with cold methanol and co-immunostained using monoclonal antibodies against cleaved ABHD16A-C and Lamin-B1. Data are shown as mean ± SD (*n* = 3 for AKI groups, *n* = 2 for *Tak1*$^{fl/fl}$ and *Tak1*$^{tamIEC\text{-}KO}$ groups). Statistical significance was determined using one-way ANOVA with Sidak's multiple comparison test. Scale bars, 20 μm.