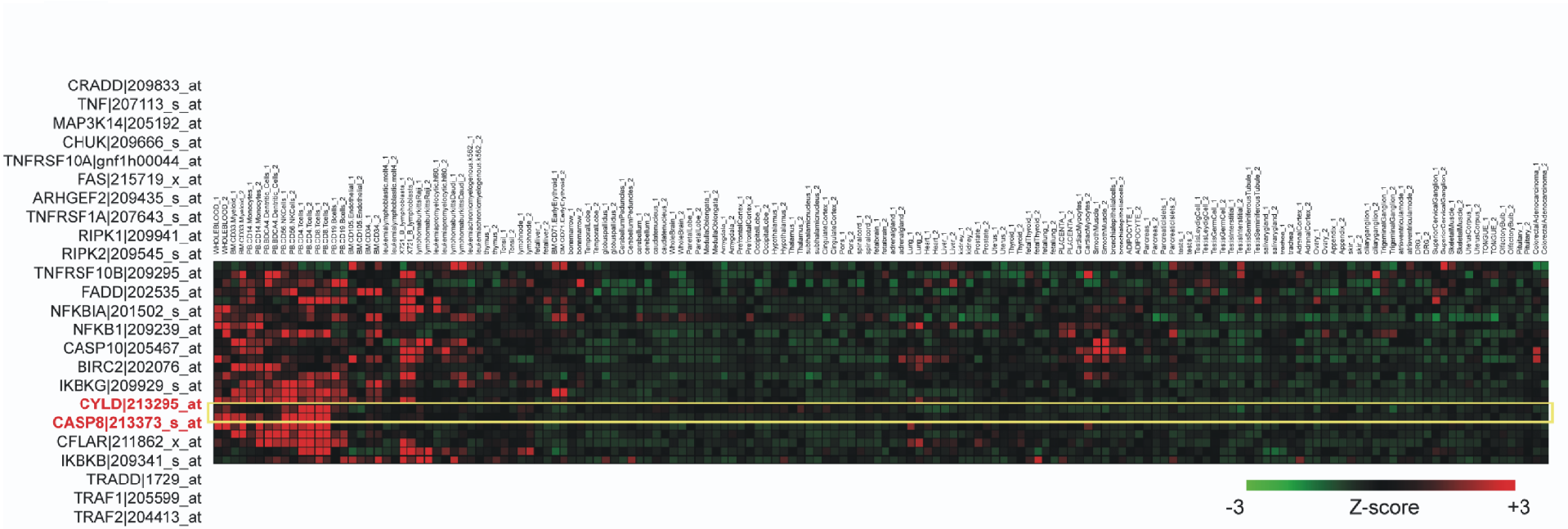
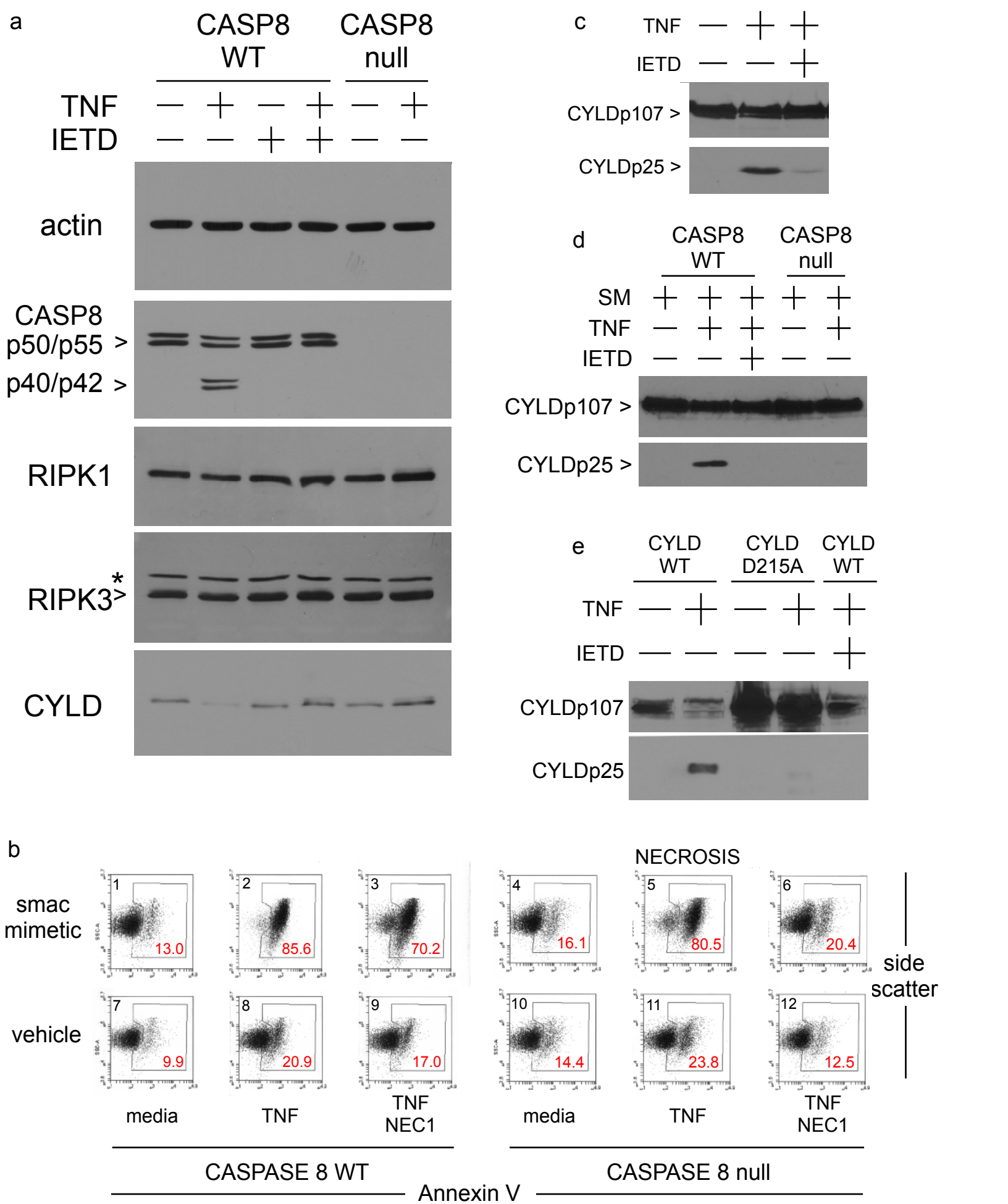
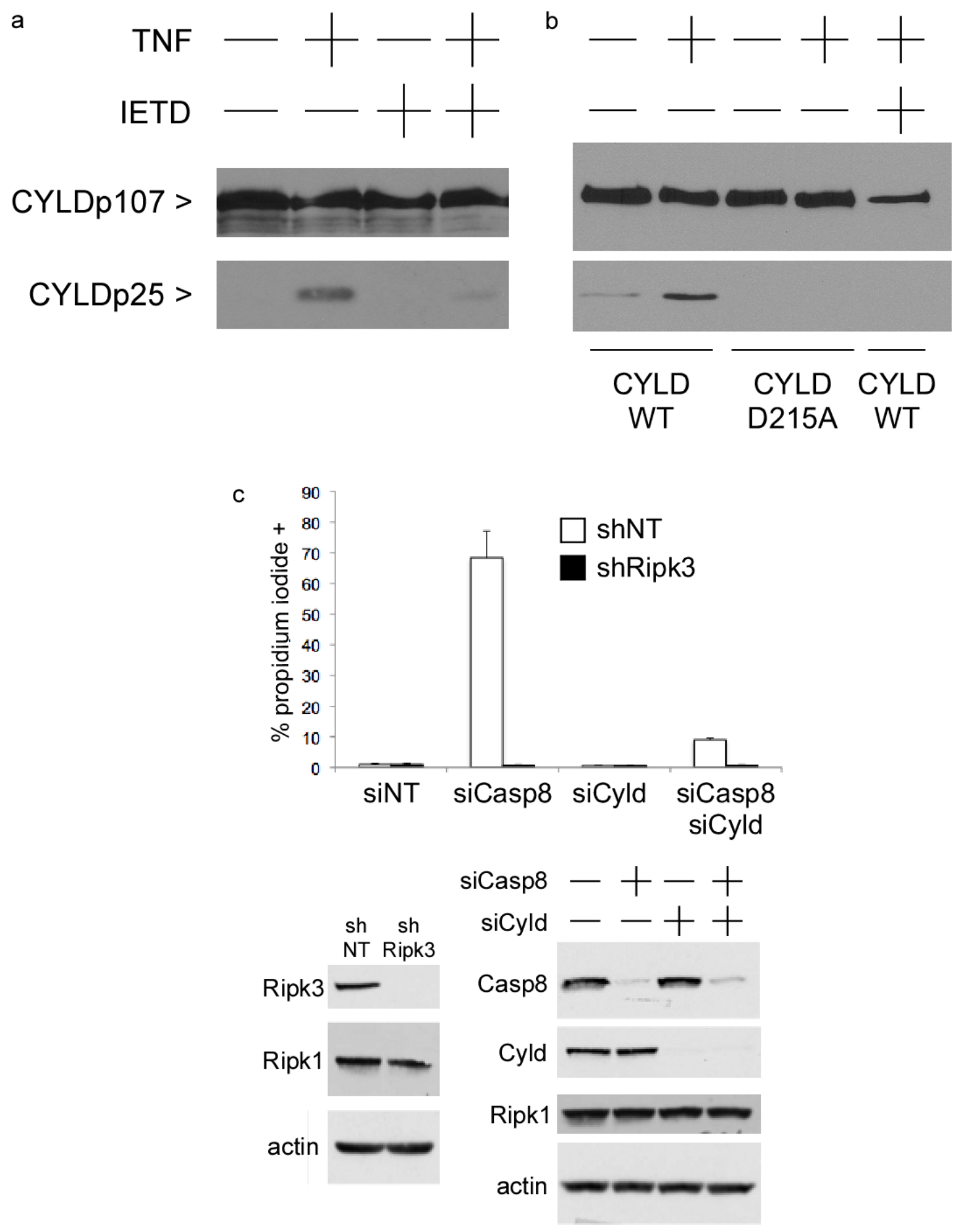


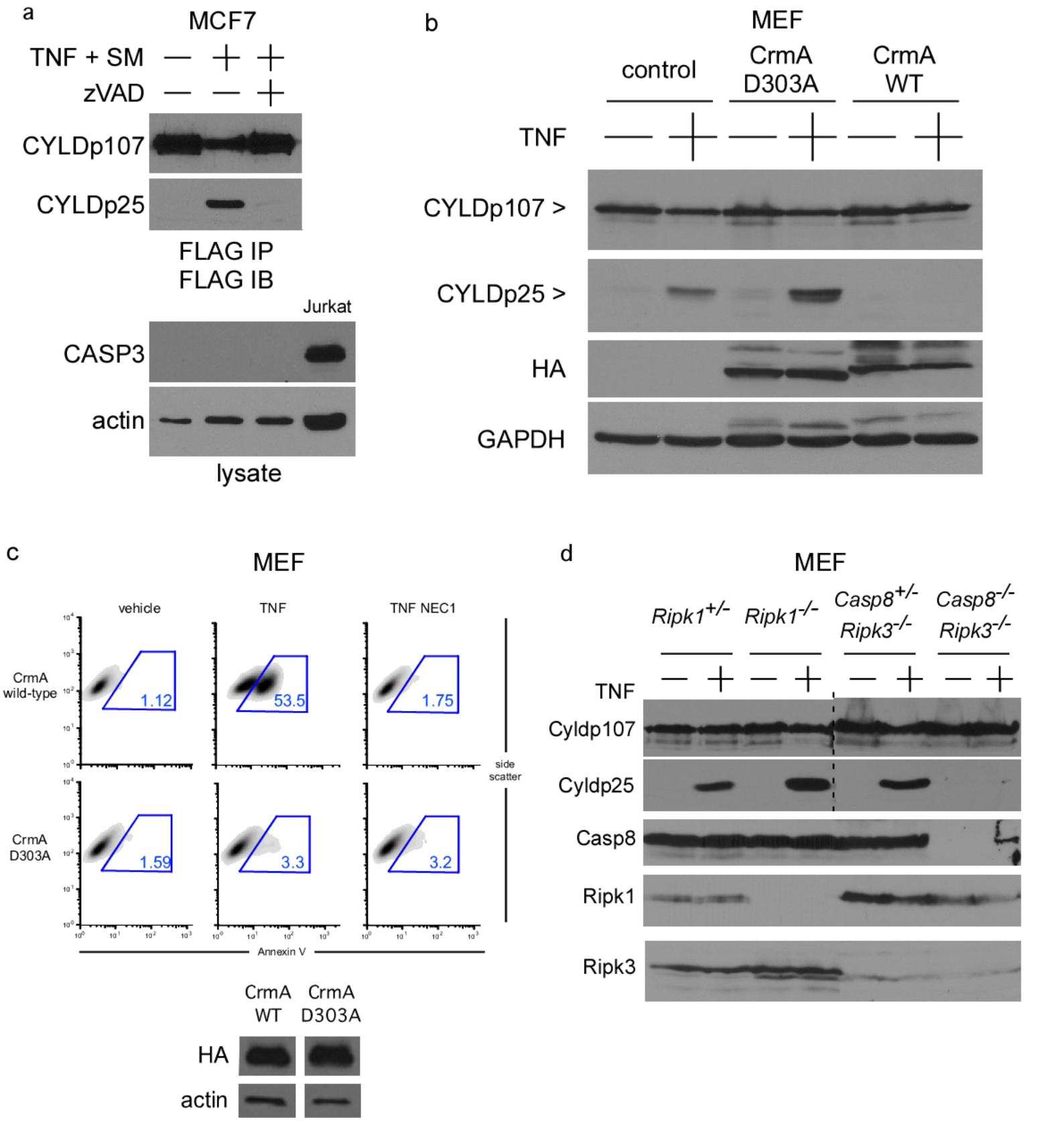
Supplementary Figure 1



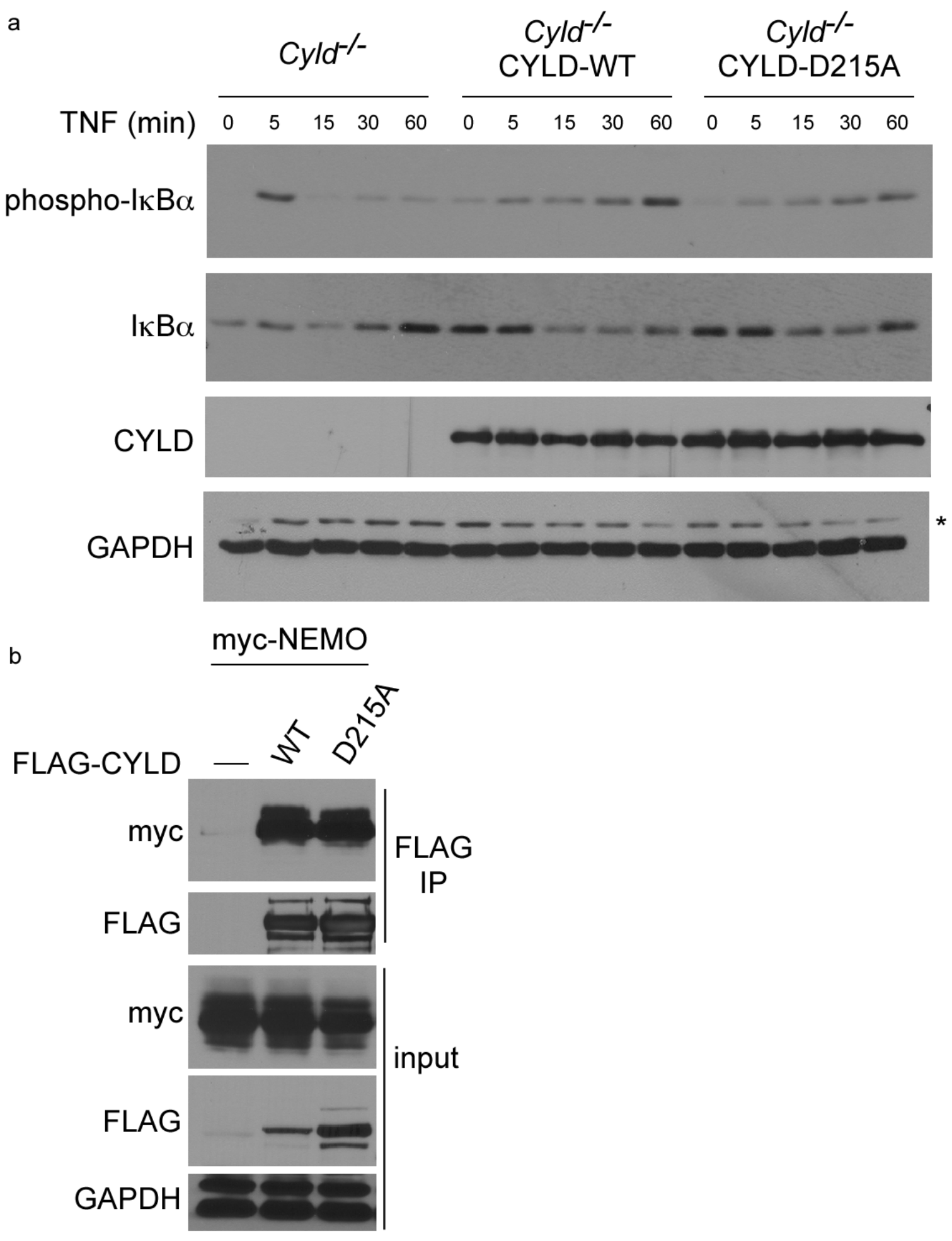
Supplementary Figure 2



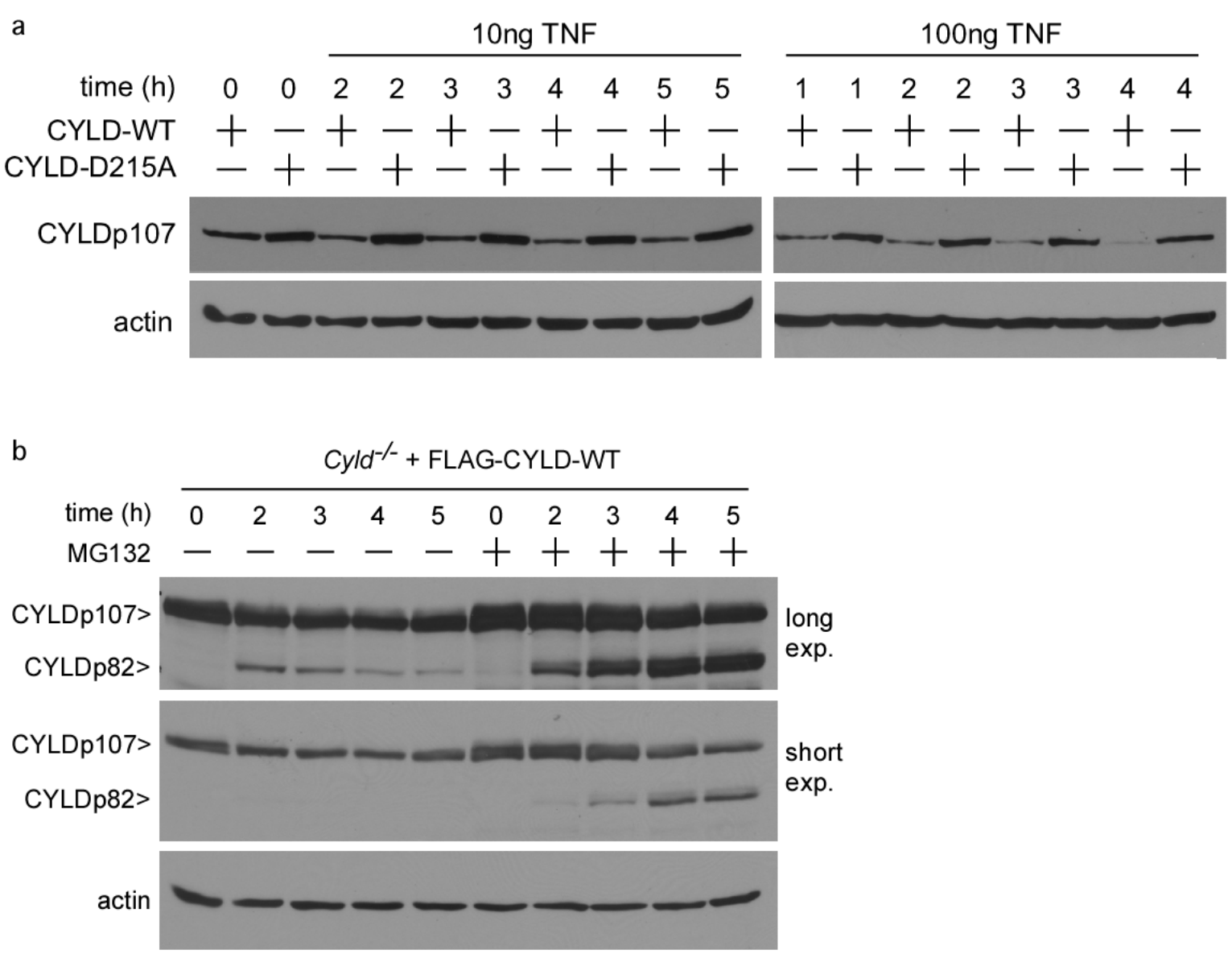
Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6

Supplementary Figure Legends

Supplementary Figure 1

***CYLD* and *CASPASE 8* genes are co-regulated**

Analysis of gene expression across 79 tissues was carried out as described previously [Ref: PMID: 18636086]. Briefly, microarray data files were obtained from the Novartis GNF human expression atlas version 2 resource [Ref: PMID: 15075390], and expression values of probe sets from the HG-U133A (Affymetrix, Santa Clara, CA, USA) platform and the GNF1H custom chip were analyzed. The data set was normalized by using global median scaling and we filtered the data by excluding from the analysis probe sets with 100% 'absent' calls (MAS 5.0 algorithm) across all 79 tissues. CYLD and CASPASE 8 mRNA levels are up-regulated in peripheral blood leukocytes, suggesting co-regulation.

Supplementary Figure 2

CYLD is processed at aspartate 215 by active CASPASE 8 in Jurkat T cells.

(a) Jurkat T cells deficient in CASPASE 8 (clone I9.2, CASP8 null) and the parental cell line (clone A3, CASP8 WT) were pre-treated with 100 nM SMAC mimetic for four hours. Triton-soluble cell lysates were obtained after 6 hours stimulation with 10 ng/ml human TNF with or without 50 μM IETD-fmk as indicated, and immunoblotted sequentially with antibody specific for endogenous CYLD, CASPASE 8, RIPK1, RIPK3 and actin (loading control). CASPASE 8 autocleavage products (p40/p42) are shown, > highlights RIPK3 band, * is a non-specific band. (b) Cell death in CASPASE 8 deficient Jurkat T cells was measured by Annexin V staining and flow cytometry 24 hours after stimulation with 10 ng/ml human TNF, with or without SMAC mimetic pre-treatment in the presence or absence of 30 μM Necrostatin-1 (NEC1). The number in the polygon gate is the percentage of cells that stain with Annexin V. The data confirm that CASP8-WT Jurkat T cells preferentially undergo apoptosis when pro-survival signaling is blocked by SMAC mimetic i.e. inhibition of RIPK1 kinase activity by Necrostatin-1 had very little effect on cell death responses of CASP8-WT cells (panel 2 versus panel 3). In contrast, all cell death occurring in CASP8-null T cells is blocked by Necrostatin-1

(panel 5 versus panel 6) confirming that CASPASE 8-deficient Jurkat T cells proceed directly into programmed necrosis. Therefore, CASPASE 8 activated by TNF and SMAC mimetic represses programmed necrosis and this correlates with CASPASE 8-dependent degradation of CYLD, but not the RIPK1 and RIPK3 kinases. (c) NEMO-deficient Jurkat T cells (clone 8321) were stimulated with 10 ng/ml TNF for 5 hours in the presence of 50 μM IETD-fmk. SDS-soluble lysates were blotted with antibody that recognises the N-terminus of CYLD. The unprocessed CYLDp107 and the CYLDp25 cleavage product generated from endogenous CYLD are indicated. (d) CASPASE 8-sufficient and CASPASE 8-deficient Jurkat T cells stably expressing FLAG-CYLD-WT were treated with 100 nM SMAC mimetic for one hour and then stimulated with 50 ng/ml human TNF for a further three hours in the presence of vehicle or 50 μM IETD-fmk, as indicated. FLAG-tagged CYLD proteins were immunoprecipitated from triton-soluble lysates and immunoblotted with FLAG-specific antibody to detect unprocessed CYLD (p107) and the CYLD cleavage product (p25) generated by active CASPASE 8. (e) CASPASE 8-sufficient Jurkat T cells stably expressing FLAG-CYLD-WT or FLAG-CYLD-D215A were treated with 100 nM SMAC mimetic for one hour and then stimulated with 50 ng/ml human TNF for a further three hours in the presence of vehicle or 50 μM IETD-fmk, as indicated. FLAG-tagged CYLD proteins were immunoprecipitated from triton-soluble lysates and immunoblotted with FLAG-specific antibody to detect unprocessed CYLD (p107) and the CYLD cleavage product (p25) generated by active CASPASE 8.

Supplementary Figure 3

CASPASE 8 represses CYLD and RIPK3-dependent programmed necrosis in L929 cells.

(a) L929 cells were stimulated with 100 ng/ml TNF in the presence of DMSO vehicle or 100 μM IETD-fmk. SDS-soluble lysates were immunoblotted with antibody specific for the N-terminus of CYLD; the unprocessed CYLDp107 and the CYLDp25 cleavage product generated from the endogenous CYLD protein are shown. (b) L929 cells stably expressing FLAG-CYLD-WT or FLAG-CYLD-D215A were stimulated with 50 ng/ml human TNF for five hours in the presence of vehicle or 50 μM IETD-fmk.

Caspase 8-dependent processing of FLAG-CYLD-WT but not FLAG-CYLD-D215A to generate the p25 fragment of CYLD was demonstrated by immunoprecipitation and FLAG immunoblot. This confirms that the pro-survival active Caspase 8 in TNF-treated L929 cells is capable of processing CYLD at aspartate 215. (c) L929 cells stably transfected with a non-targetting (shNT) or Ripk3-targetting hairpin (shRipk3) were transiently transfected with Caspase 8 siRNA oligos in conjunction with CYLD siRNA oligos, as indicated. Forty-eight hours post-transfection, L929 cells undergoing programmed necrosis due to depletion of endogenous Caspase 8 were quantified by propidium iodide staining and flow cytometry. The percentage of L929 cells that are permeabilised and take up propidium iodide is shown confirming that depletion of endogenous Caspase 8 in L929 cells triggers programmed necrosis in a Cyld and Ripk3-dependent manner. Error bars indicate standard deviation of the mean of triplicate samples. Samples of lysates were immunoblotted with antibody specific for endogenous Ripk3, Caspase 8, Cyld, Ripk1 and actin (loading control) to verify efficient knockdown of each protein.

Supplementary Figure 4

CrmA blocks processing of CYLD whereas CASPASE 3, RIPK1 and RIPK3 are not required.
(a) CASPASE 3 deficient human breast cancer cell line MCF7 stably expressing FLAG-CYLD were treated with 50 ng/ml TNF with smac mimetic pretreatment. FLAG-tagged CYLD was immunoprecipitated and immunoblotted with FLAG-specific antibody to detect unprocessed CYLDp107 and the cleavage product CYLDp25. A sample of the corresponding whole cell lysates were blotted for CASPASE 3 to confirm the complete absence of CASPASE 3 protein in the MCF7 cells; a sample of protein lysate from Jurkat T cells was used as a positive control. (b) *Cyld*$^{-/-}$ MEFs stably expressing FLAG-CYLD-WT were transduced with retrovirus encoding a control protein (bacterial glutathione-s-transferase), CrmA-WT or the point mutant CrmA-D303A, which is unable to inhibit active CASPASE 8. MEFs were stimulated with 10 ng/ml TNF for 4 hours and whole cell lysates were immunoblotted with FLAG-specific antibody to detect unprocessed CYLDp107 and the

cleaved product CYLDp25 and then reblotted with HA-specific antibody to confirm expression of the CrmA constructs. (c) Wild-type MEFs were transduced with control protein, CrmA-WT or CrmA-D303A as described in (b) and then stimulated with 10 ng/ml TNF with or without 30 μM Necrostatin-1 (NEC1) as indicated. The percentage of Annexin V + cells obtained by flow cytometry after 24 hours TNF stimulation is shown inside the polygon gate. Western blot confirms equivalent expression of the CrmA proteins. (d) *Ripk1*$^{+/+}$, *Ripk1*$^{-/-}$, *Casp8*$^{+/-}$*Ripk3*$^{-/-}$, *Casp8*$^{-/-}$*Ripk3*$^{-/-}$ MEFs were stimulated with 100 ng/ml TNF for 5 hours. Whole cell lysates were immunoblotted with antibody specific for the N-terminal of CYLD. The unprocessed CYLDp107 protein and the cleavage product CYLDp25 generated from the endogenous proteins are indicated. Immunoblots were sequentially probed for Caspase 8, Ripk1 and Ripk3 to confirm knockout.

Supplementary Figure 5

Activation of NFκB is similar between CYLD-WT and CYLD-D215A MEFs.

(a) *Cyld*$^{-/-}$ MEFs and *Cyld*$^{-/-}$ MEFs stably expressing CYLD-WT and CYLD D215A were stimulated with 100 ng/ml TNF for the time-periods indicated. Cytoplasmic protein lysates were immunoblotted with antibody against phospho- IκBα, total IκBα, CYLD and GAPDH (loading control). (b) HEK 293EBNA cells were transiently transfected with 5μg of plasmid encoding myc-NEMO, FLAG-CYLD-WT or FLAG-CYLD-D215A. FLAG-tagged proteins were immunoprecipitated 24 hours post transfection and immunoblotted with myc-specific antibody to demonstrate co-precipitation of CYLD and NEMO. A sample of the lysate was blotted for myc and FLAG to confirm equivalent expression of the constructs.

Supplementary Figure 6

TNF triggers loss of the deubiquitinase domain of CYLD-WT but not D215A.

(a) *Cyld*$^{-/-}$ MEFs stably expressing FLAG-tagged CYLD-WT and CYLD-D215A were stimulated with either 10 or 100 ng/ml TNF for the time-periods indicated. Cytoplasmic protein lysates were

immunoblotted with antibody against FLAG and actin (loading control). (b) *Cyld*$^{-/-}$ MEFs stably expressing CYLD-WT were treated for 30 minutes with vehicle or 25μM MG132 and then stimulated with 10 ng/ml TNF for the time-periods indicated. Cytoplasmic protein lysates were immunoblotted with antibody against the C-terminus of CYLD and actin (loading control).