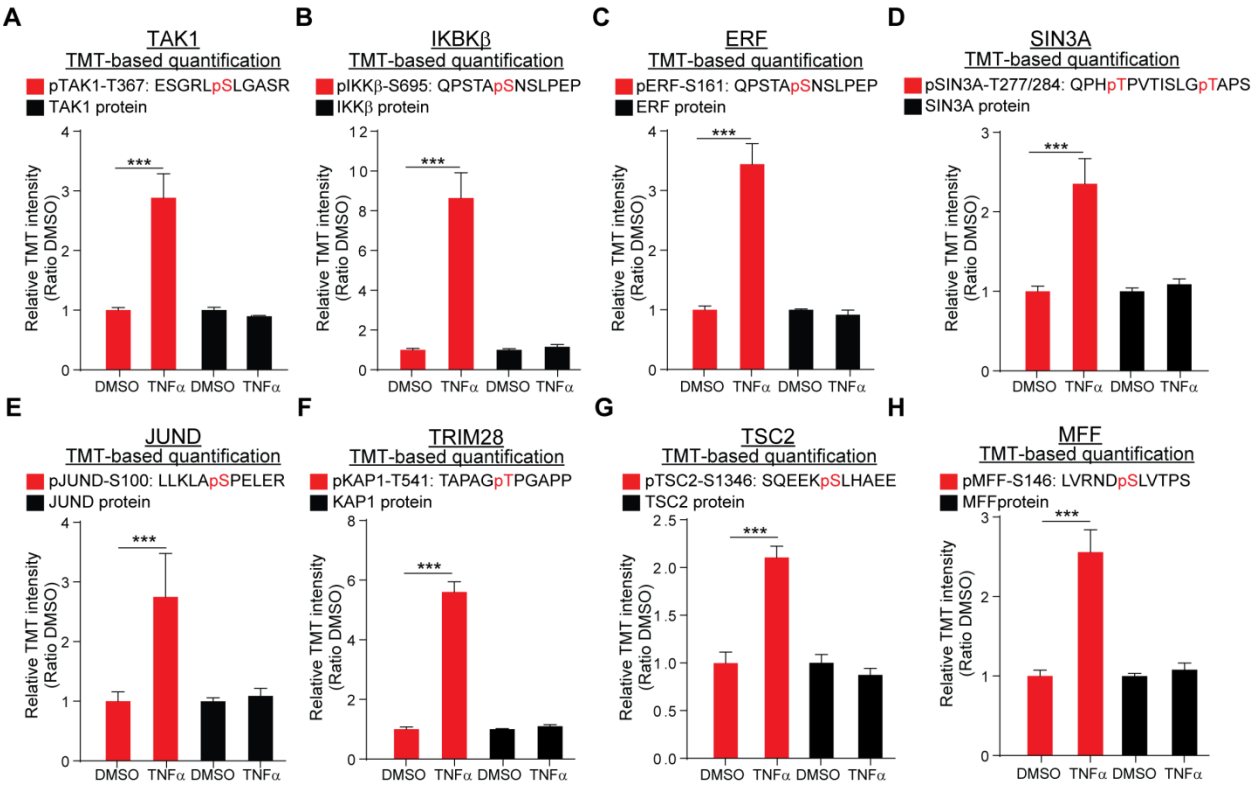
Supplementary information

Quantitative Phospho-proteomic Analysis of TNFα/NFκB Signaling Reveals a Role for RIPK1 Phosphorylation in Suppressing Necrotic Cell Death

Firaz Mohideen, Joao A. Paulo, Alban Ordureau, Steve P. Gygi, and J. Wade Harper

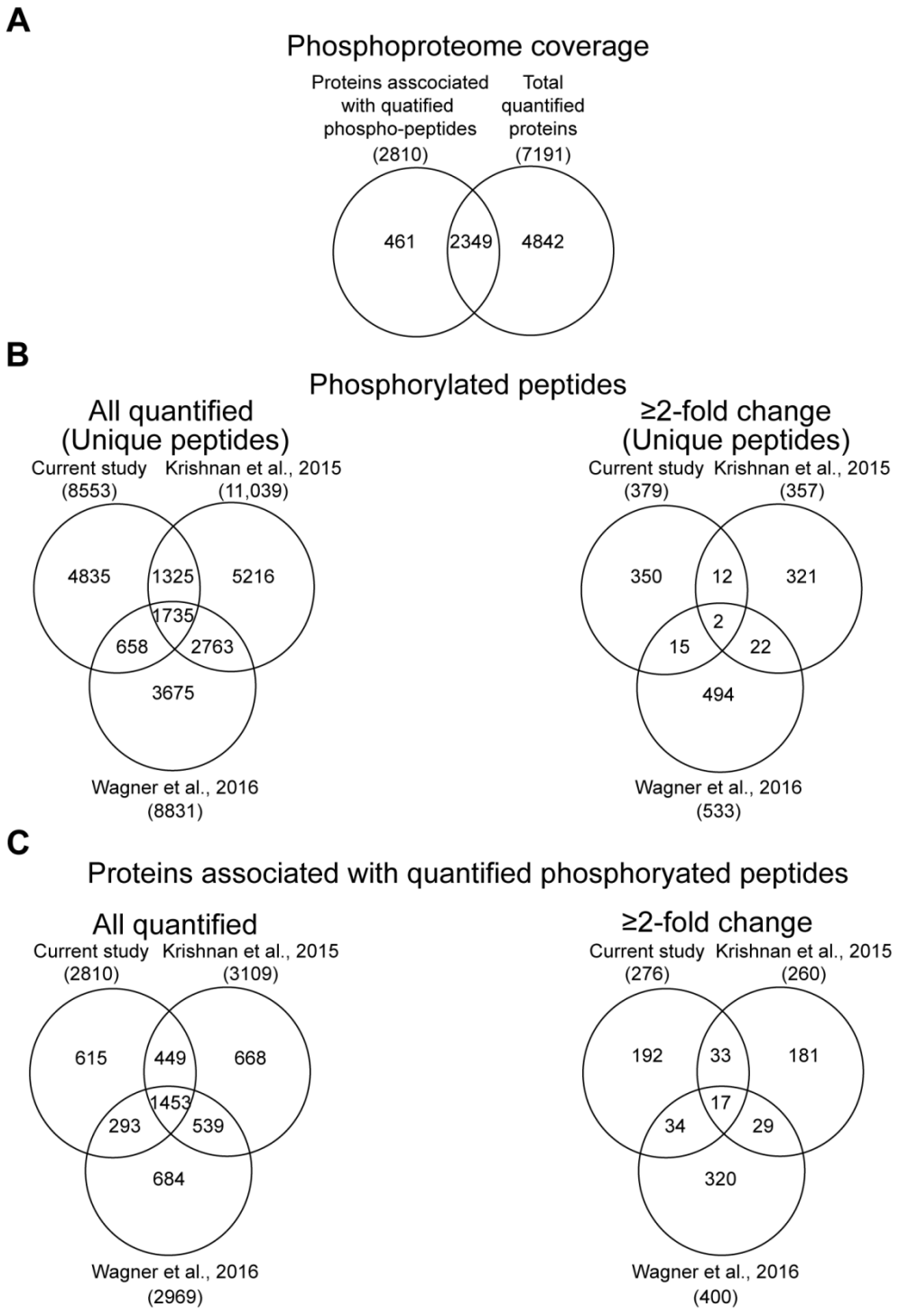
Supplemental Figure 1



Supplemental Figure 1: Representative phosphorylated peptides demonstrating altered abundances in response to TNFα stimulation (*A*) Relative TMT signal to noise intensities across the DMSO control-treated and TNFα-treated H1299 cells for the indicated TAK1 phosphorylated peptide (red bars) and the TAK1 protein (black bars). (*B*) As in (*A*), but for IKKβ. (*C*) As in (*A*), but for ERF. (*D*) As in (*A*), but for SIN3A. (*E*) As in (*A*), but for JUND. (*F*) As in (*A*), but for TRIM28. (*G*) As in (*A*), but for TSC2. (*H*) As in (**A**), but for MFF.

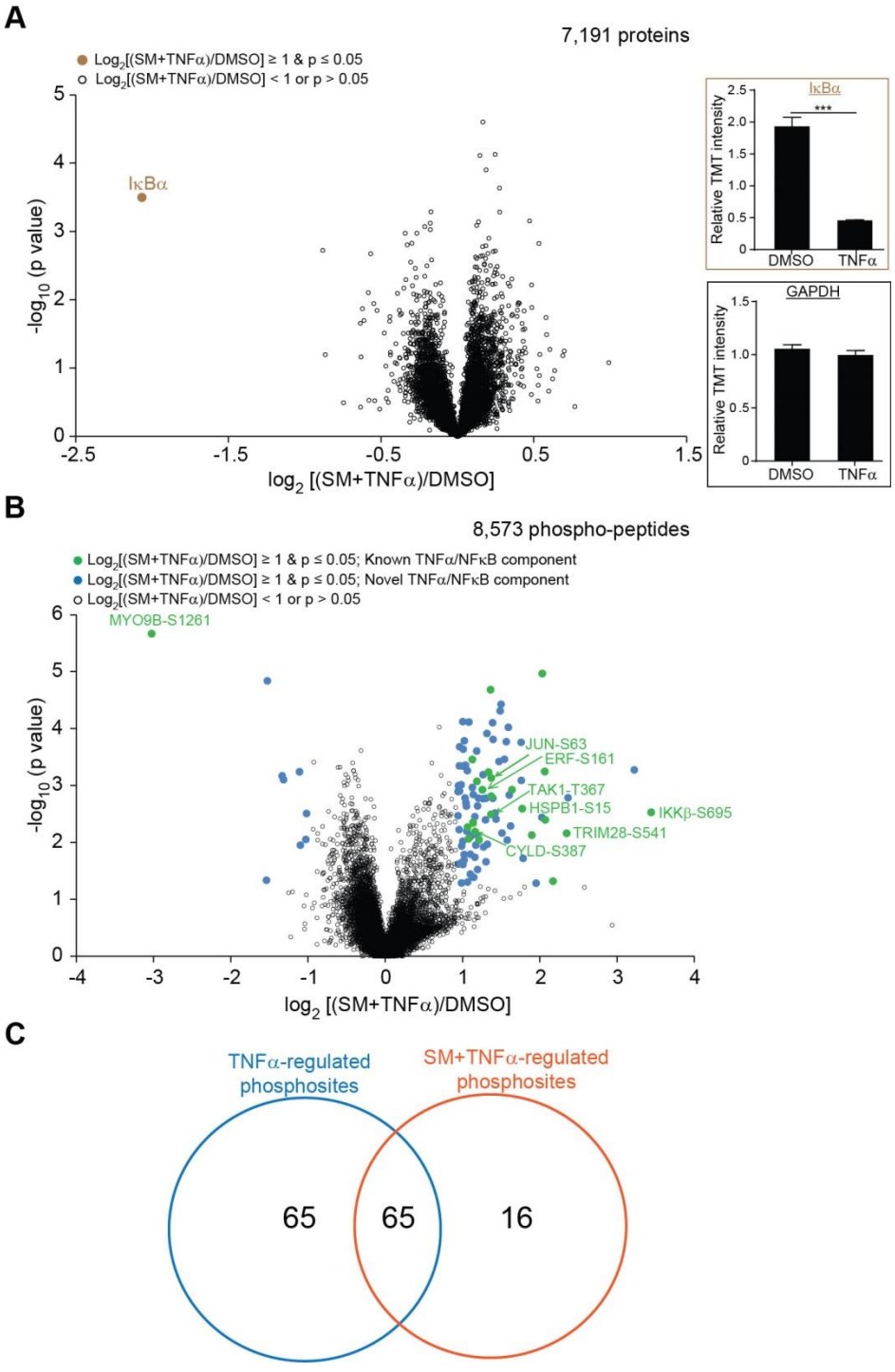
Error bars represent quadruplicate measurements for DMSO and triplicate measurements for TNFα + s.e.m. Data were analyzed by Student's t-test, ***$p \leq 0.001$; **$p \leq 0.01$; *$p \leq 0.05$.

Supplemental Figure 2



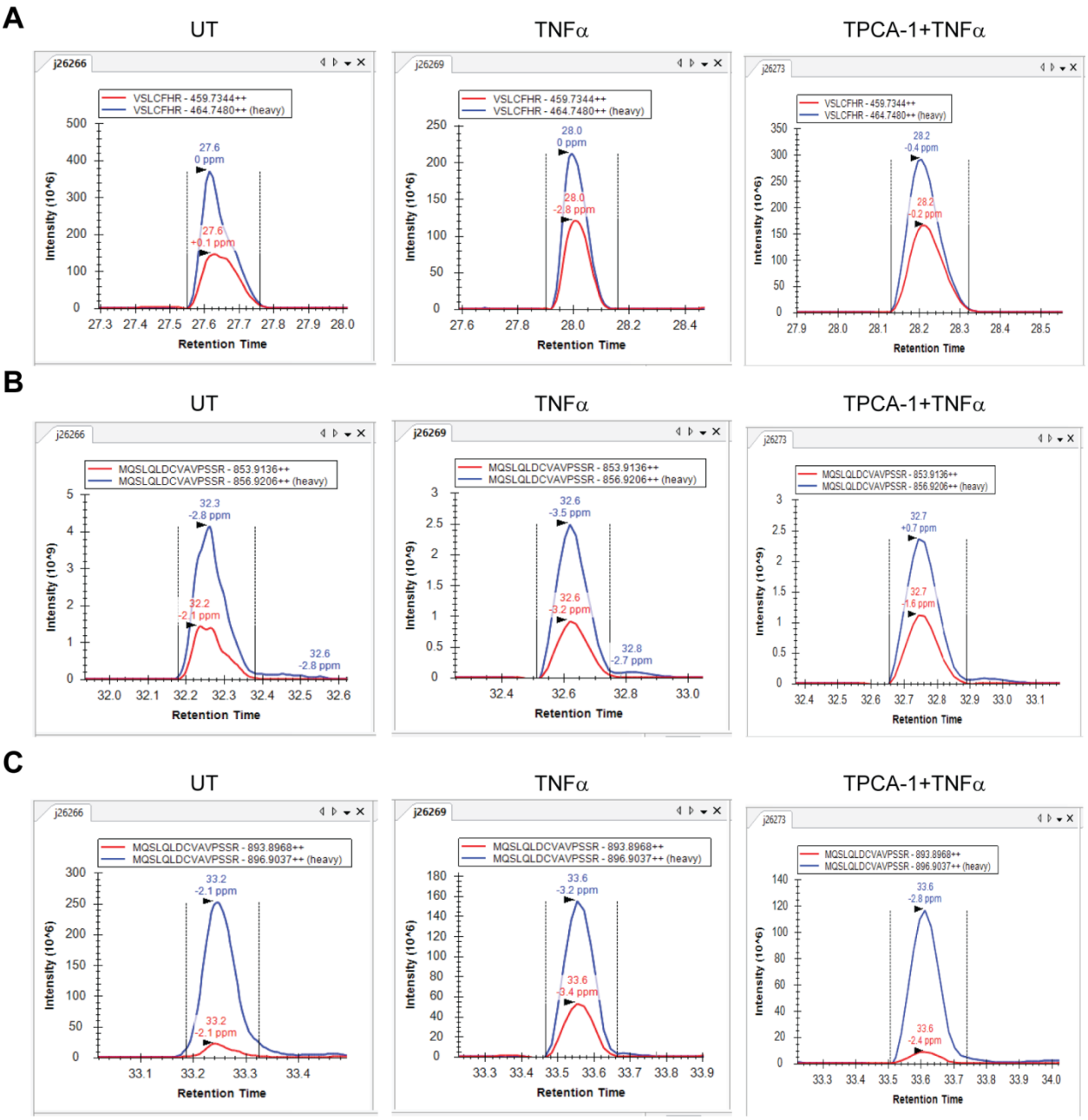
Supplemental Figure 2: Number of quantified phosphorylated peptides and their associated proteins Venn diagrams representing the overlap between (*A*) proteins associated with quantified phosphorylated peptides and the total quantified proteins, (*B*) the unique quantified phosphorylated peptides in the current study and those quantified in two previous studies. All unique quantified phosphorylated peptides (left) and phosphorylated peptides displaying changes of 2-fold or more (right), (*C*) the proteins associated with the quantified phosphorylated peptides in the current study and those quantified in two previous studies. Proteins associated with all quantified phosphorylated peptides (left) and phosphorylated peptides displaying changes of 2-fold or more (right).

Supplemental Figure 3



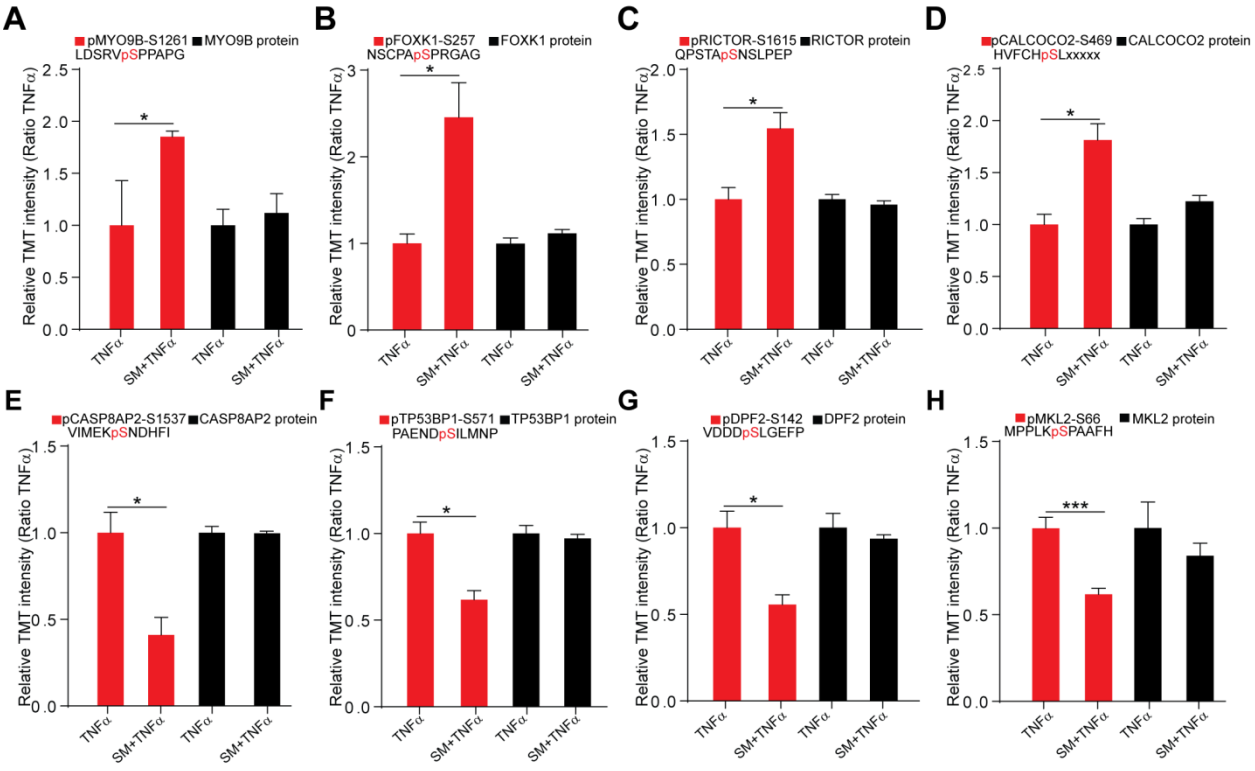
Supplemental Figure 3: Analysis of the SM+TNFα regulated proteome and phosphoproteome as quantified by TMT-based mass spectrometry (*A*) Volcano plot highlighting statistically significant (≥2-fold; $p \leq 0.05$) changes in protein abundances in SM+TNFα-treated cells compared to the DMSO control (Brown filled circle). Insets: Relative TMT signal to noise intensities across the control treated and SM+TNFα-treated samples for IκBα (top) and GAPDH (bottom) protein abundances. (*B*) As in (*A*), but highlighting statistically significant (≥2-fold; $p \leq 0.05$) changes in phosphorylated peptide abundances in SM+TNFα-treated cells compared to the DMSO control. Color code is as described in Fig. 2*E*. (*C*) Venn diagram representing the overlap between TNFα-regulated phosphosites with and without SM
Error bars represent quadruplicate measurements for DMSO and triplicate measurements for (SM+TNFα) + s.e.m. Data were analyzed by Student's t-test, ***$p \leq 0.001$.

Supplemental Figure 4



Supplemental Figure 4: Representative extracted signals for the RIPK1 heavy labelled (blue) and endogenous (red) peptides for the AQUA-PRM experiment: (*A*) ^{31}VSL*C*FHR^{37}, (*B*) ^{318}*M*QSLQLD*C*VAVPSSR^{332}, and (*C*) ^{318}*M*QpSLQLD*C*VAVPSSR^{332}. Also see Experimental Procedures and supplemental Table 5.

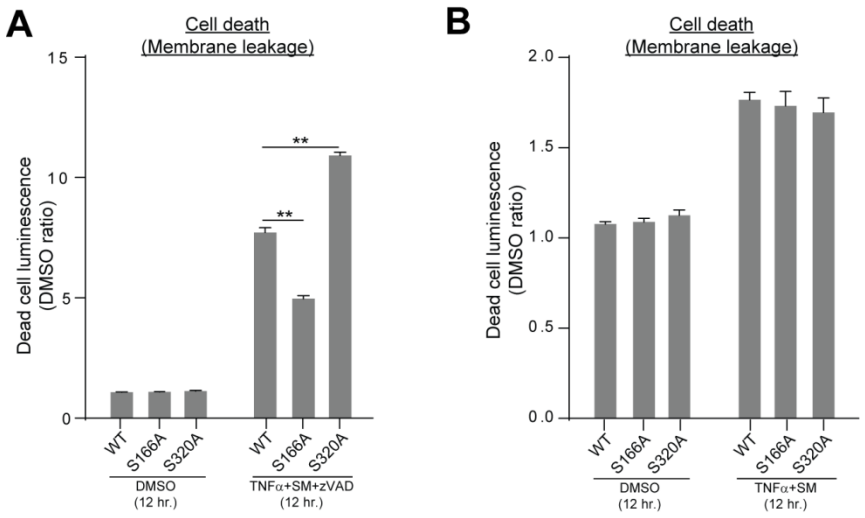
Supplemental Figure 5



Supplemental Figure 5: Representative phosphorylated peptides demonstrating altered abundances between TNFα stimulation and (SM+ TNFα) stimulation (*A*) Relative TMT signal to noise intensities across the TNFα-treated and (SM+TNFα)-treated H1299 cells for the indicated MYO9B phosphorylated peptide (red bars) and the MYO9B protein (black bars). (*B*) As in (*A*), but for FOXK1. (*C*) As in (*A*), but for RICTOR. (*D*) As in (*A*), but for CALCOCO2. (*E*) As in (*A*), but for CASP8AP2. (*F*) As in (*A*), but for TP53BP1. (*G*) As in (*A*), but for DPF2. (*H*) As in (*A*), but for MKL2.

Error bars represent triplicate measurements + s.e.m. Data were analyzed by Student's t-test, ***$p \leq 0.001$; *$p \leq 0.05$.

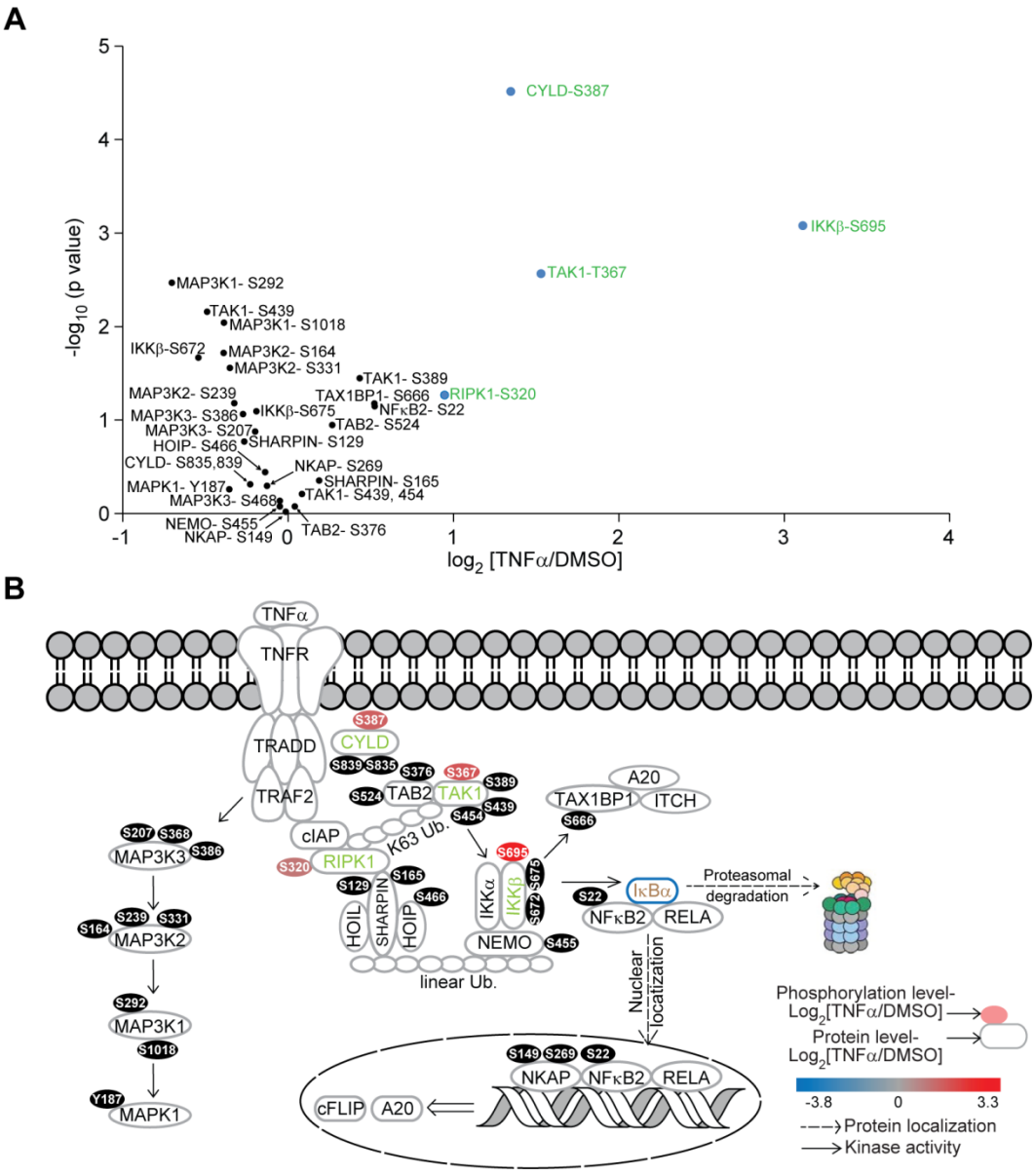
Supplemental Figure 6



Supplemental Figure 6: Ser166 and Ser320 on RIPK1 are important for necrotic, but not apoptotic cell death (*A*) Jurkat RIPK1$^{-/-}$ cells reconstituted with the indicated cHF-RIPK1 isoforms were control-treated or treated for 30 minutes with zVAD and SM prior to 12 hr TNFα exposure. The dead cell luminescence was measured using the CytoTox-Glo kit (Promega). (*B*) As in (*A*), but the indicated cell lines were control-treated or treated for 30 minutes with SM prior to 12 hr TNFα exposure.

Error bars represent triplicate measurements + s.e.m. Data were analyzed by Student's t-test, $^{**}p \leq 0.01$

Supplemental Figure 7



Supplemental Figure 7: Quantified phosphorylation sites of central TNFα/NFκB/MAPK components. (*A*) Volcano plot highlighting changes in phosphorylated peptide abundances among central components of the TNFα/NFκB/MAPK pathway in TNFα-treated cells compared to the DMSO control. Blue filled circles represent phosphorylated peptides displaying statistically significant (≥2-fold; $p \leq 0.05$) changes. (*B*) Data from the literature was utilized to display the central components of the signaling pathway. Proteins containing phosphorylation sites displaying significant (≥2-fold; p≤0.05) changes in abundances were annotated with their phosphorylation sites, color-coded according to the $\log_2$ fold change in TNFα-treated cells compared to the DMSO control. Phosphorylation sites displaying no significant changes (<2-fold and/or p>0.05) in abundances between TNFα-treated and DMSO control are colored in black. Color code for Gene symbols are as described in Fig. 2*E*.

Supplementary Table 1: Peptides identified in the 10-plex TMT experiment in DMSO-treated (n=4), TNFα-treated (n=3), and SM+TNFα-treated (n=3) H1299 cells. The protein accession number, gene symbol, assigned peptide sequence, precursor charge and mass/charge, number of matched and unmatched ions, the peptide redundancy, number of missed cleavages, and the link to spectra are included.

Supplementary Table 2: Proteins identified in the 10-plex TMT experiment in DMSO-treated (n=4), TNFα-treated (n=3), and SM+TNFα-treated (n=3) H1299 cells. The protein accession number, molecular weight, and protein sequence coverage % are included.

Supplementary Table 3: Proteins quantified in the 10-plex TMT experiment in DMSO-treated (n=4), TNFα-treated (n=3), and SM+TNFα-treated (n=3) H1299 cells. Indicated are the normalized and scaled summed signal to noise for each of the 10 channels (126 to 131), ratio of average TNFα to DMSO signal to noise intensity, $\log_2$ ratio of average TNFα to DMSO signal to noise intensity, *p*-values of the Student's t-test for each TNFα to DMSO signal to noise intensity, ratio of average SM+TNFα to DMSO signal to noise intensity, $\log_2$ ratio of average SM+TNFα to DMSO signal to noise intensity, *p*-values of the Student's t-test for each SM+TNFα to DMSO signal to noise intensity, ratio of average TNFα to (SM+ TNFα) signal to noise intensity, $\log_{1.5}$ ratios of average TNFα to (SM+ TNFα) signal to noise intensity, *p*-values of the Student's t-test for each TNFα to (SM+ TNFα) signal to noise intensity.

Supplementary Table 4: Phosphorylated peptides identified in the 10-plex TMT experiment in DMSO-treated (n=4), TNFα-treated (n=3), and SM+TNFα-treated (n=3) H1299 cells. The protein accession number, gene symbol, assigned peptide sequence, precursor charge and mass/charge, number of matched and unmatched ions, the peptide redundancy, number of missed cleavages, the AScore(s) for the phosphorylated site(s), and the link to spectra are included.

Supplementary Table 5: Phosphorylated peptides quantified in the 10-plex TMT experiment in DMSO-treated (n=4), TNFα-treated (n=3), and SM+TNFα-treated (n=3) H1299 cells. Included are the normalized and scaled summed signal to noise for each of the 10 channels (126 to 131), ratio of average TNFα to DMSO signal to noise intensity, $\log_2$ ratios of average TNFα to DMSO signal to noise intensity, *p*-values

of the Student's t-test for each TNFα to DMSO signal to noise intensity, ratio of average SM+TNFα to DMSO signal to noise intensity, $\log_2$ ratios of average SM+TNFα to DMSO signal to noise intensity, *p*-values of the Student's t-test for each SM+TNFα to DMSO signal to noise intensity, tatio of average TNFα to (SM+ TNFα) signal to noise intensity, $\log_{1.5}$ ratios of average TNFα to (SM+ TNFα) signal to noise intensity, *p*-values of the Student's t-test for each TNFα to (SM+ TNFα) signal to noise intensity.

Supplementary Table 6: Quantified phosphorylated peptides and their associated proteins. Sheet 1: Overlapping proteins between the proteome and phosphoproteome datasets; Sheet 2: Overlapping phosphorylation sites between the current study and two previous SILAC-based quantifications (1, 2) of the TNFα response (all unique quantified peptides); Sheet 3: Overlapping phosphorylation sites between the current study and two previous SILAC-based quantifications (1, 2) of the TNFα response (unique quantified peptides displaying changes of 2-fold or more). Peptides quantified in at least two replicates of TNFα-treatment in the previous studies were used for the comparison.

Supplementary Table 7: Quantified phosphorylated peptides and their associated proteins. Overlapping proteins associated with quantified phosphorylated peptides between the current study and the two previous SILAC-based quantifications of the TNFα response. Sheet 1: Proteins associated with all quantified peptides; Sheet 2: Proteins associated with quantified peptides displaying changes of 2-fold or more. Peptides quantified in at least two replicates of TNFα-treatment in the previous studies were used for the comparison.

Supplementary Table 8: AQUA-based quantification of RIPK1 peptides. Included are the AQUA peptide sequences, AQUA peptide amount injected (µl), and the amount quantitated (fmol)- Columns E-G: Untreated, replicates 1-3; H-J: TNFα, replicates 1-3; K-M: SM+TNFα, replicates 1-3.

References

1. Krishnan, R. K., Nolte, H., Sun, T., Kaur, H., Sreenivasan, K., Looso, M., Offermanns, S., Krüger, M., and Swiercz, J. M. (2015) Quantitative analysis of the TNF-α-induced phosphoproteome reveals AEG-1/MTDH/LYRIC as an IKKβ substrate. *Nat. Commun.* 6, 6658
2. Wagner, S. A., Satpathy, S., Beli, P., and Choudhary, C. (2016) SPATA2 links CYLD to the TNF-α receptor signaling complex and modulates the receptor signaling outcomes. *EMBO J.* 35, 1–17