Figure S1. Reponses of WT, TRAF6 KO, Flag-KI TRAF6 , NEMO KO, Flag-NEMO reconstituted NEMO KO and Flag-KI MyD88 RAW 264.7 cell lines to LPS treatment. **A-E.** The cell lines were stimulated for the indicated periods with LPS. Total lysates were used for immunoblotting. MAPK activation was detected by anti phospho-JNK, phospho-p38 and phospho-ERK antibodies. NF-κB activation was detected by anti phospho-p65 and IκB antibody. **F.** TNFα secretion by the indicated cell lines treated with or without LPS for 12hr was measured by ELISA.

Figure S2. Quality of quantitative data of MyD88 dataset. **A.** Correlation analysis of protein intensities between any two samples. The matrix of correlation plots is shown, and the colors represent the indicated correlation coefficients. **B.** Quantitative reproducibility of protein intensities in biological triplicates. **C.** Dynamic range of all identified proteins in MyD88 dataset. The log10 abundance of proteins calculated using "TOP 3" approach in ten time-points are shown. Some interactors as well as the bait protein are highlighted.

Figure S3. Quality of quantitative data of NEMO dataset. **A.** Correlation analysis of protein intensities between any two samples. The matrix of correlation plots is shown, and the colors represent the indicated correlation coefficients. **B.** Quantitative reproducibility of protein intensities in biological triplicates. **C.** Dynamic range of all identified proteins in NEMO dataset. The log10 abundances of proteins calculated using "TOP 3" approach in ten timepoints are shown. Some interactors as well as the bait protein are highlighted.

Figure S4. Identification of high-confidence interactors of MyD88. **A.** Heatmap of protein abundances in LPS treated samples relative to non-treated samples. Hierarchical clustering analysis revealed that one cluster of proteins were differentially expressed. **B.** Differential expression analysis in MyD88 dataset. Proteins with Log_2(fold change) >1 and -Log_{10}(P-value) >1.5 were considered significantly changed. Upregulated proteins were labeled in red, and downregulated proteins were labeled in blue. **C.** Comparison of band intensities in Western blot and protein intensities in MyD88 IP SWATH-MS. The panel above showed Western blot results of IRAK1, TRAF6, TNAP3 and MyD88 in MyD88 IP samples, and the panel below showed comparison of TRAF6, TNAP3 and IRAK1 band intensities and these protein intensities in SWATH-MS. The coefficient of correlation was shown.

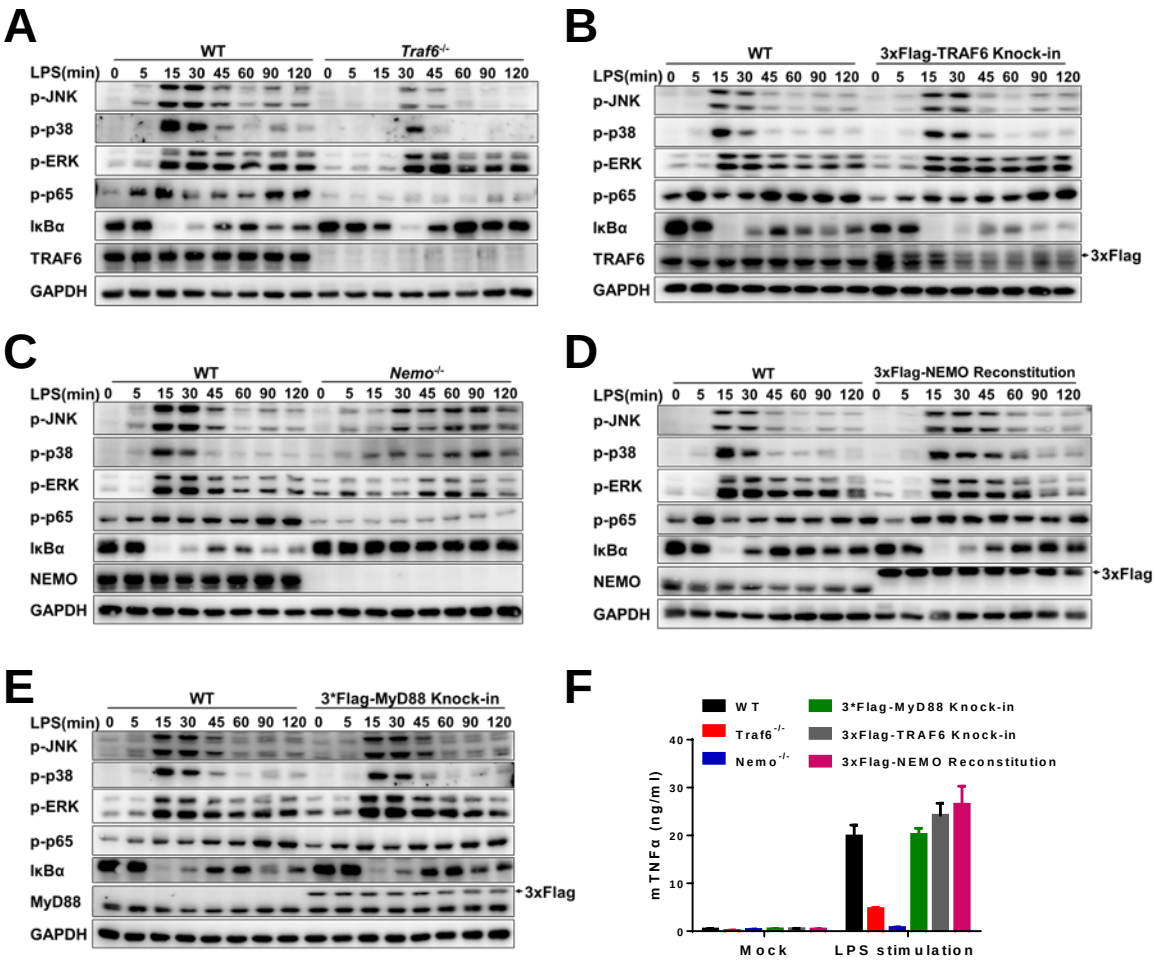
Figure S5. Identification of high-confidence interactors of NEMO. **A.** Heatmap of protein abundances in LPS treated samples relative to non-treated samples. Hierarchical clustering analysis revealed that one cluster of proteins were differentially expressed. **B.** Differential expression analysis in NEMO dataset. Proteins with Log_2(fold change) >1 and -Log_{10}(P-value) >1.5 were considered significantly changed. Upregulated proteins were labeled in red, and downregulated proteins were labeled in blue. **C.** Comparison of band intensities in Western blot and protein intensities in NEMO IP SWATH-MS. The panel above showed Western blot results of IRAK1, TNAP3, MyD88, IKKβ and NEMO in NEMO IP samples, and the panel below showed comparison of MyD88, TNAP3 and IRAK1 band intensities and these protein intensities in SWATH-MS. The coefficient of correlation was shown.

Figure S6. Measurement of some genes' mRNA by LPS stimulation and differential expression analysis in negative control dataset. **A.** Real-time RT-PCR validation of some LPS-induced genes. **B.** Differential expression analysis in negative control dataset. Proteins with Log_2(fold change) >1 and -Log_{10}(P-value) >1.5 were considered significantly changed. Upregulated proteins were labeled in red, and downregulated proteins were labeled in blue.

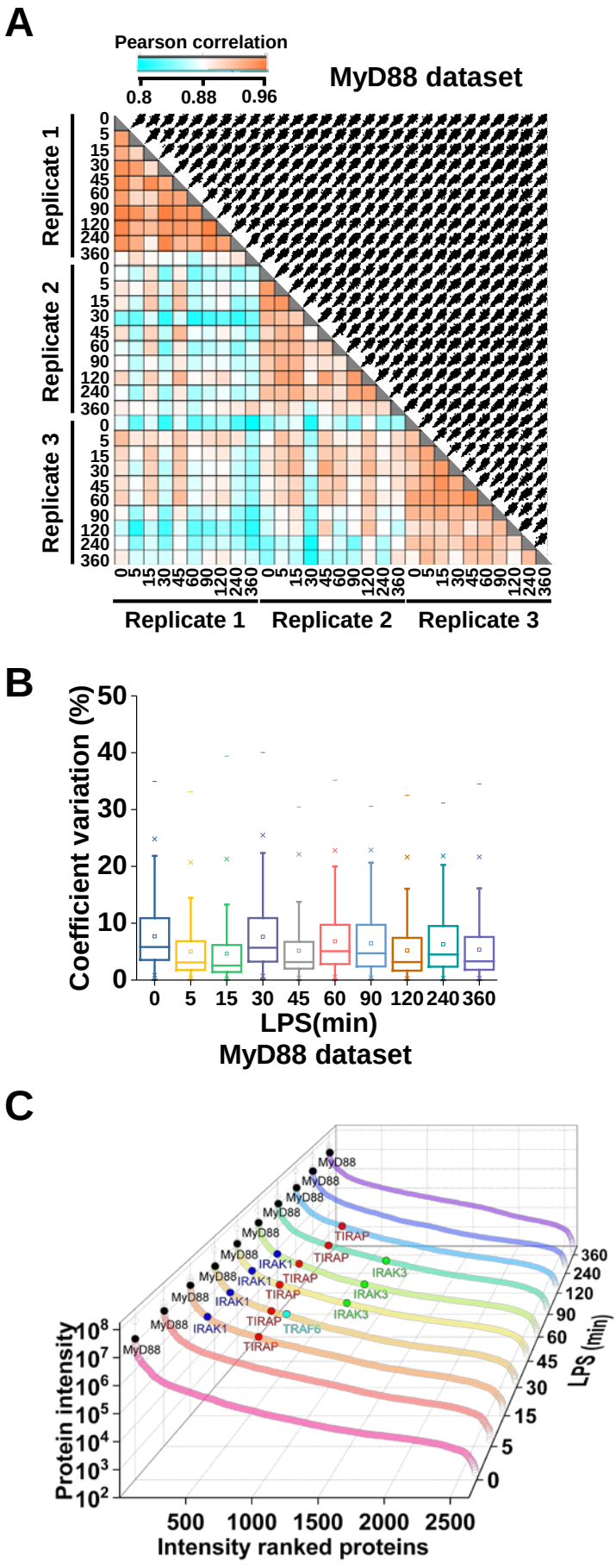
Figure S7. Luciferase assay results of some phosphosite mutants of TRAK1, TRAF6, IKKβ or

TAK1. **A-D.** 293T cells were co-transfected with the indicated constructs, NF-κB firefly luciferase reporter and renilla luciferase transfection control plasmid. Luciferase activities were measured 24hr post transfection. Values represent the fold of luciferase activity induction relative to cells transfected with empty vector.

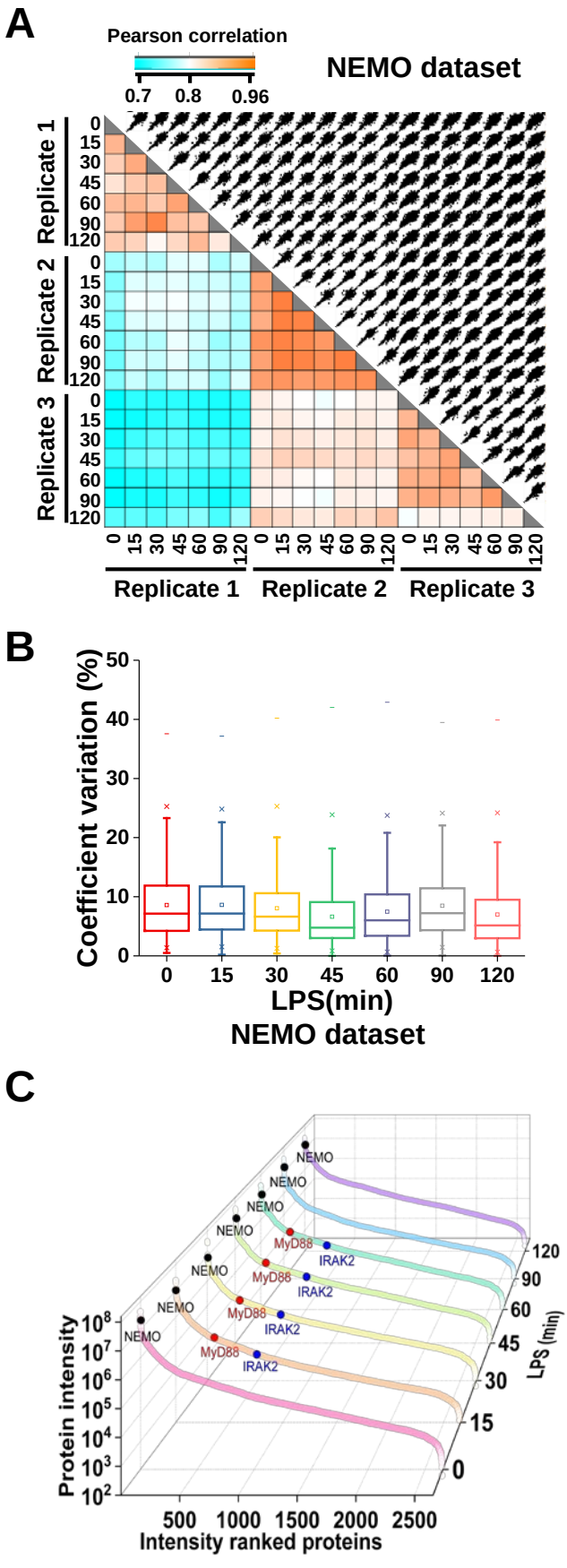
Supplementary Figure 1



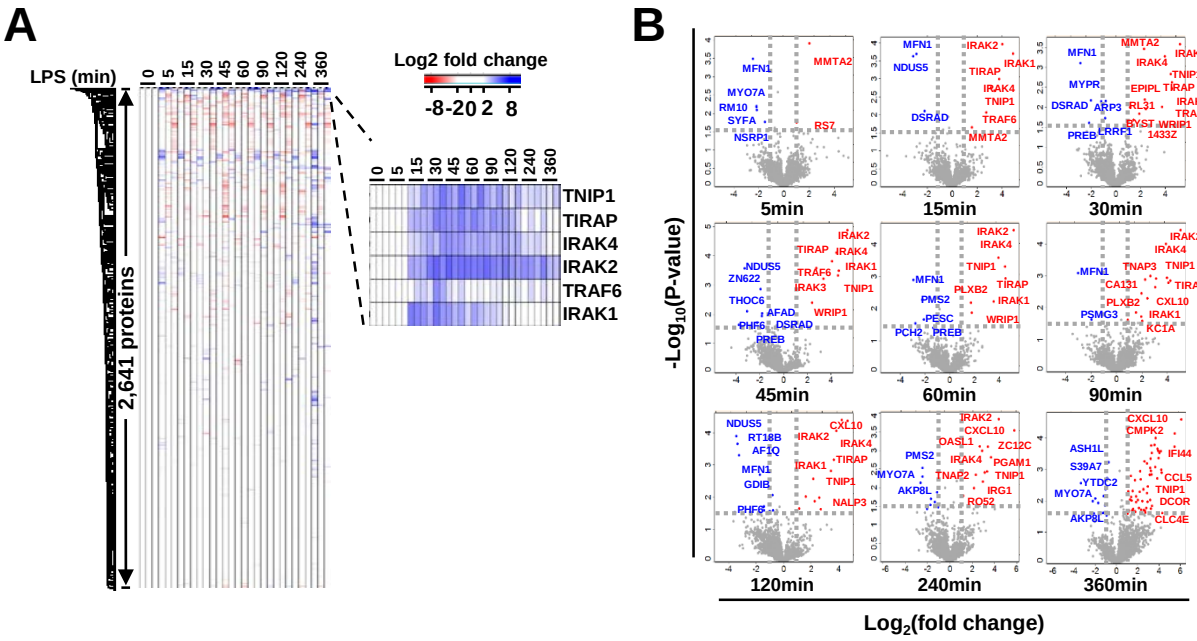
Supplementary Figure 2

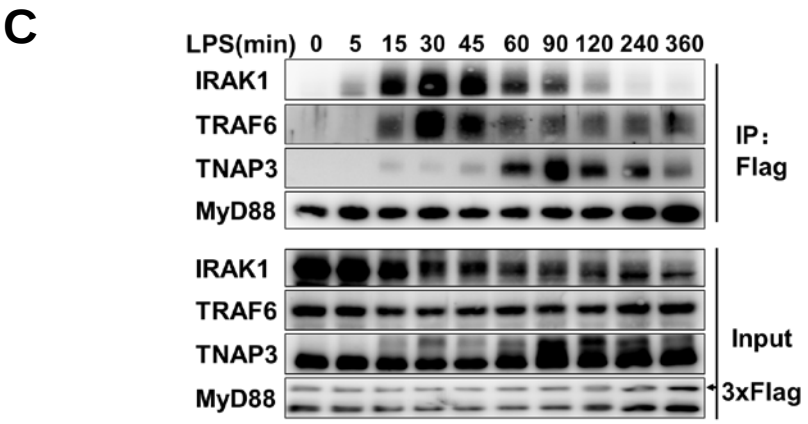


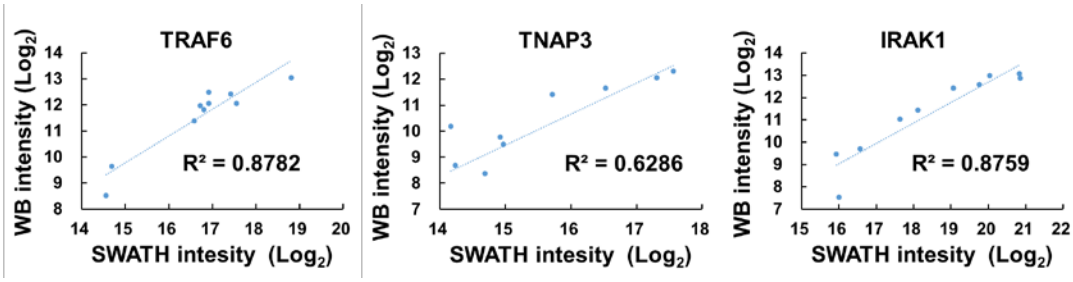
Supplementary Figure 3



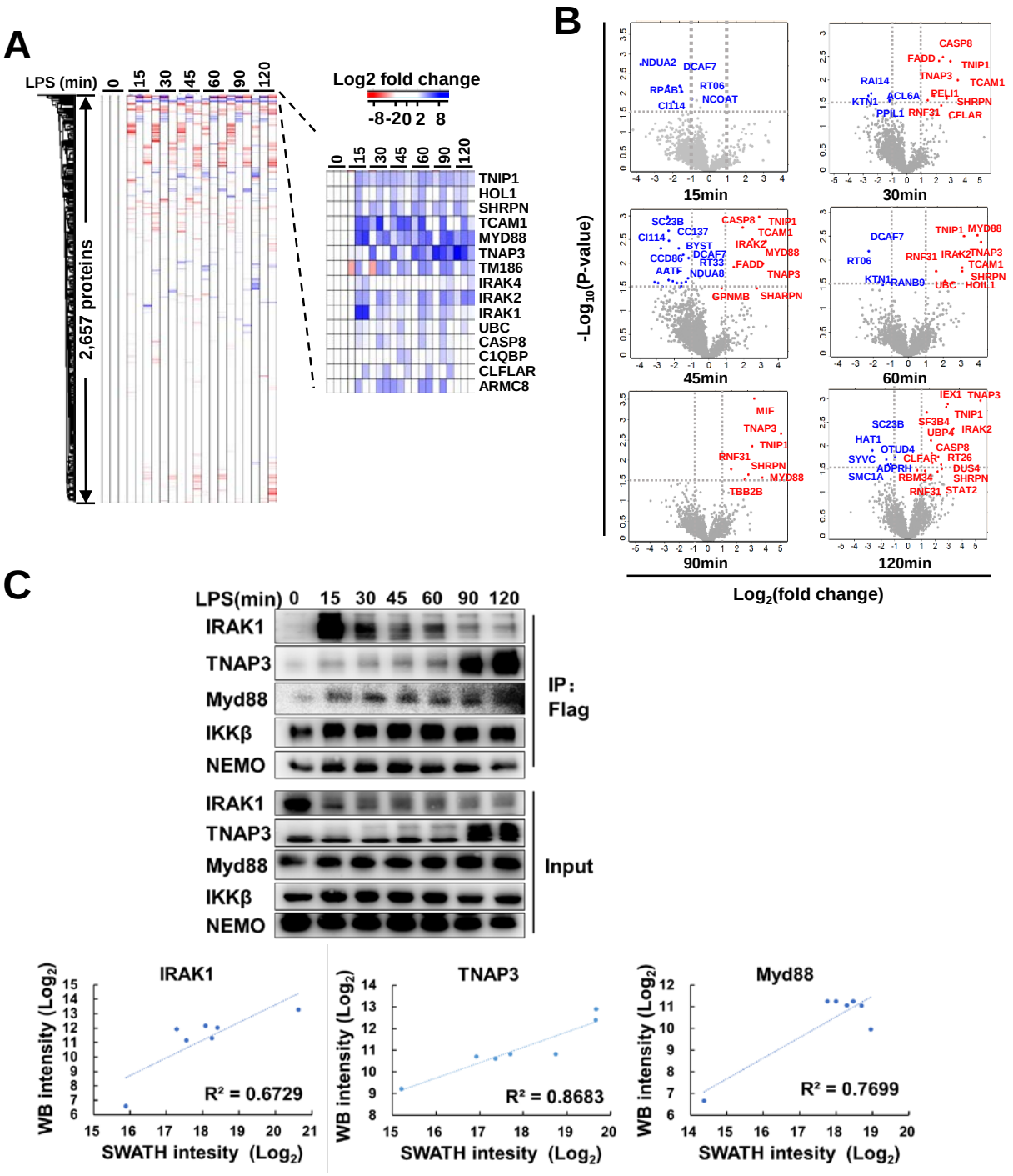
Supplementary Figure 4





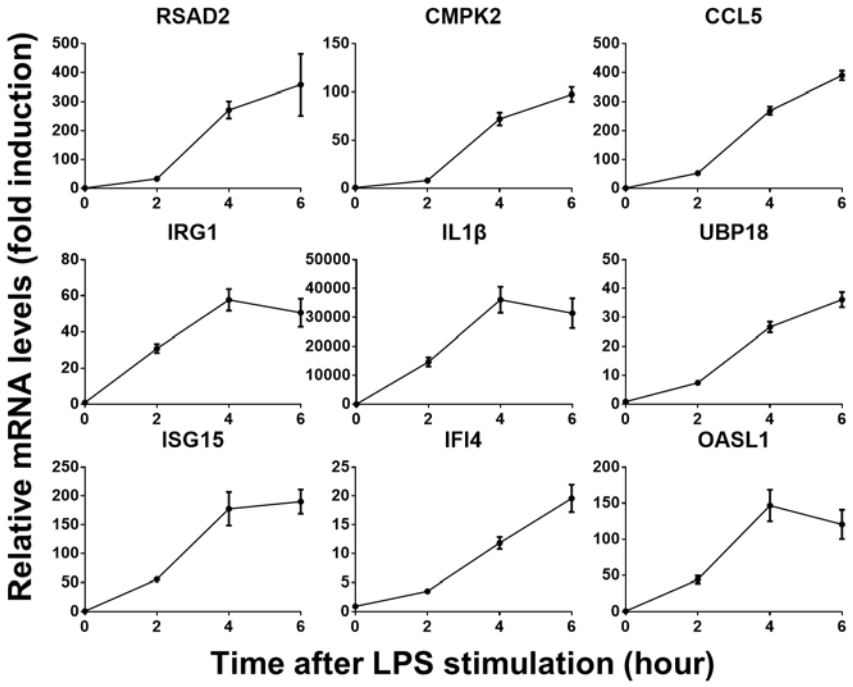


Supplementary Figure 5



Supplementary Figure 6

A



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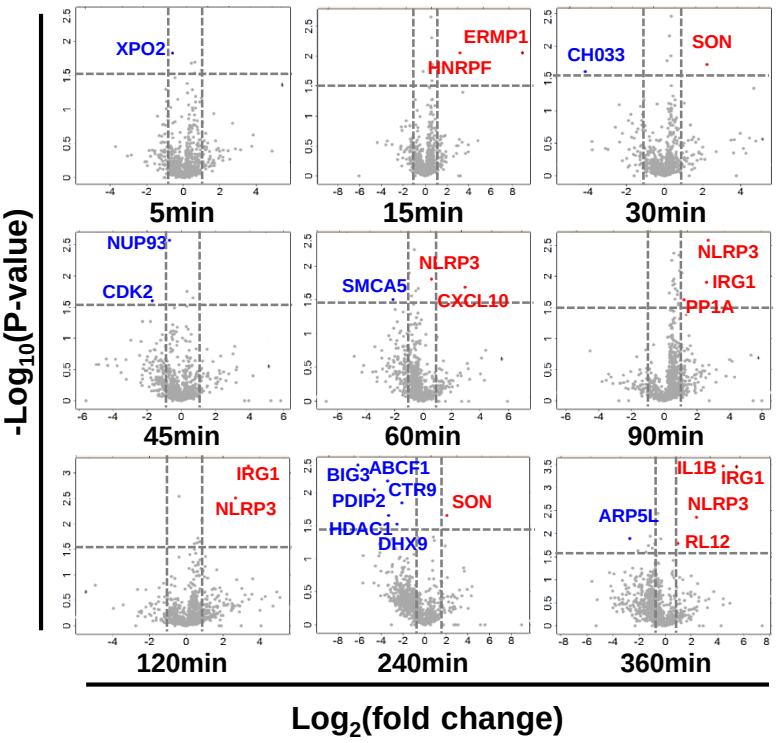

Supplementary Figure 7