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Supplemental Information

MK2 Phosphorylates RIPK1 to Prevent TNF-Induced Cell Death

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Supplementary Figures

Figure S1

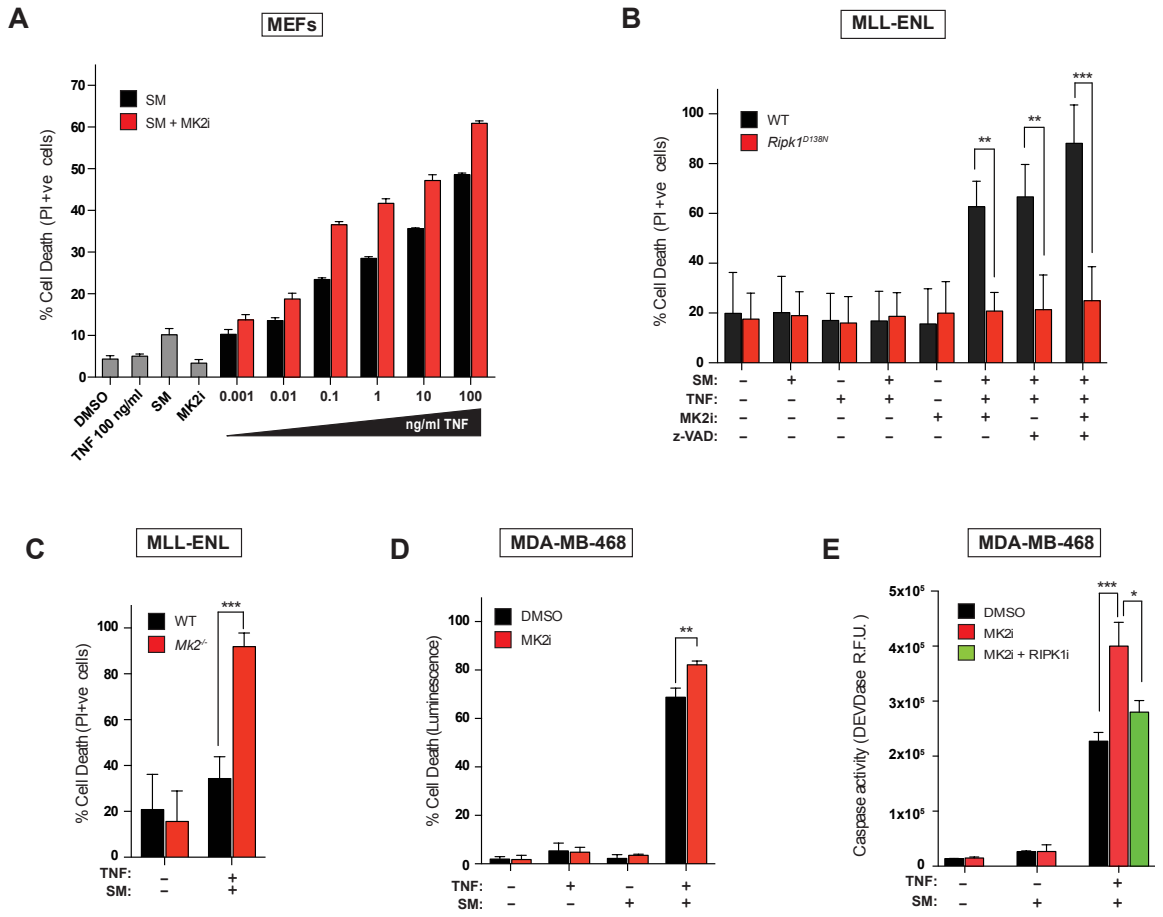

Figure S1. MK2 protects from TNF-induced cell death, Related to Figure 1

(A) Quantification of PI positive cells of primary MEFs treated with the indicated agents. Cells were pre-treated with MK2i (1 μM). **(B)** Quantification of PI positive cells of WT and *Ripk1*^{D138N} MLL-ENL cells. Cells were treated with the indicated agents for 24 hrs. TNF (10 ng/ml), SM (25 nM), MK2i (2 μM). **(C)** Quantification of PI positive cells of WT and *Mk2*$^{-/-}$ MLL-ENL cells, treated with the indicated agents for 24 hrs. TNF (10 ng/ml), SM (25 nM). **(D)** Quantification of cell viability in MDA-MB-468 cells, treated with the indicated agents. Cells were pre-treated with MK2i (1 μM). **(E)** DEVDase activity analysis of MDA-MB-468 cells left untreated or treated with the indicated agents for 5 hrs. Cells were pre-treated with MK2i (1 μM) and RIPK1i (100 nM) for 30 min. Graphs show mean ± SEM, n = 3 independent biological repeats. *p < 0.05, **p < 0.01, and ***p < 0.001.

Figure S2

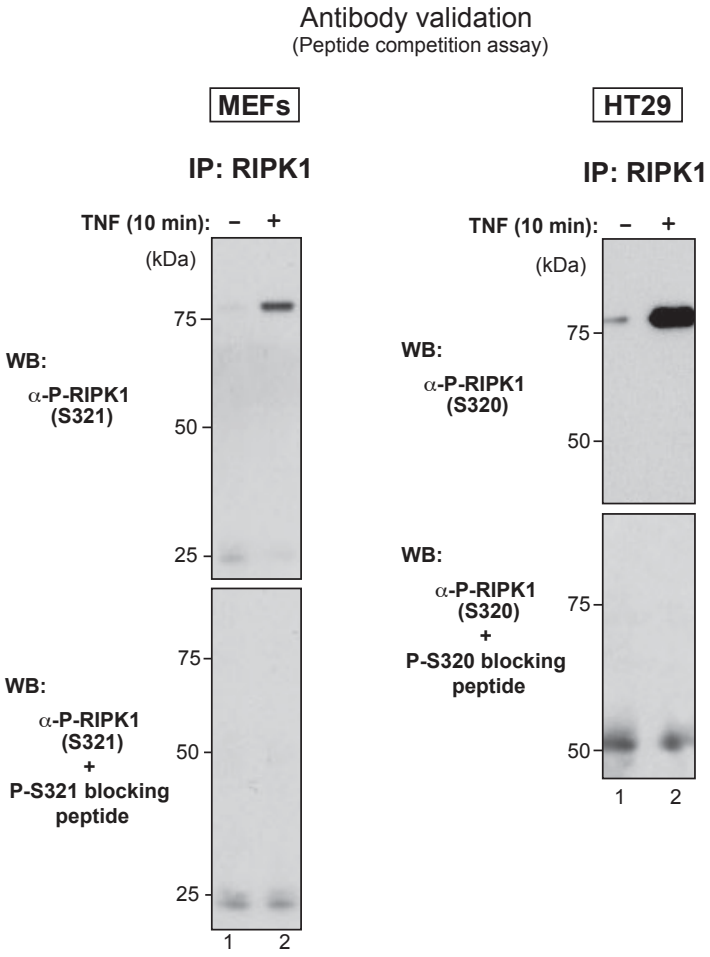

Figure S2. Antibody validation, Related to Figure 2

Western blot analysis of RIPK1 immunoprecipitates from MEFs or HT29 cells using the indicated antibodies. Cells were stimulated with TNF (10 ng/ml) for 10 min. Antibodies were either left untreated or pre-incubated with blocking P-peptide, against which these antibodies were raised against.

Figure S3

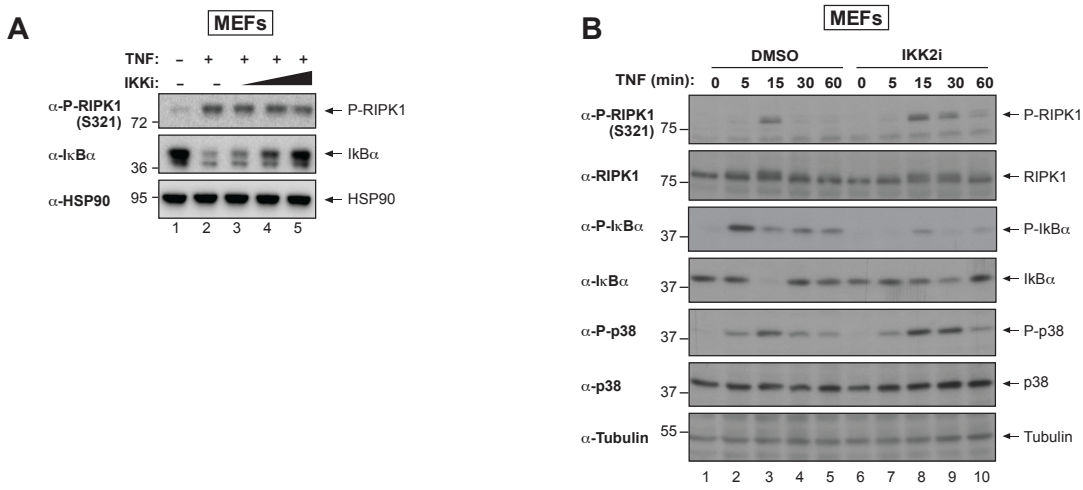

Figure S3. MK2 phosphorylates RIPK1 at S320/S321 in response to TNF stimulation, Related to Figure 3

(A) Western blot analysis of cell lysates from primary MEFs using the indicated antibodies. Cells were pre-treated with increasing concentrations of IKK2i (TCPA-1, 0.2, 1 and 5 μM) for 1 h, followed by stimulation with TNF (10 ng/ml) for 10 min. **(B)** Western blot analysis of cell lysates from primary MEFs using the indicated antibodies. Cells were pre-treated with IKK2i (BI605906, 10 μM) for 1 hr, followed by stimulation with TNF (10 ng/ml) for the indicated times.

Figure S4

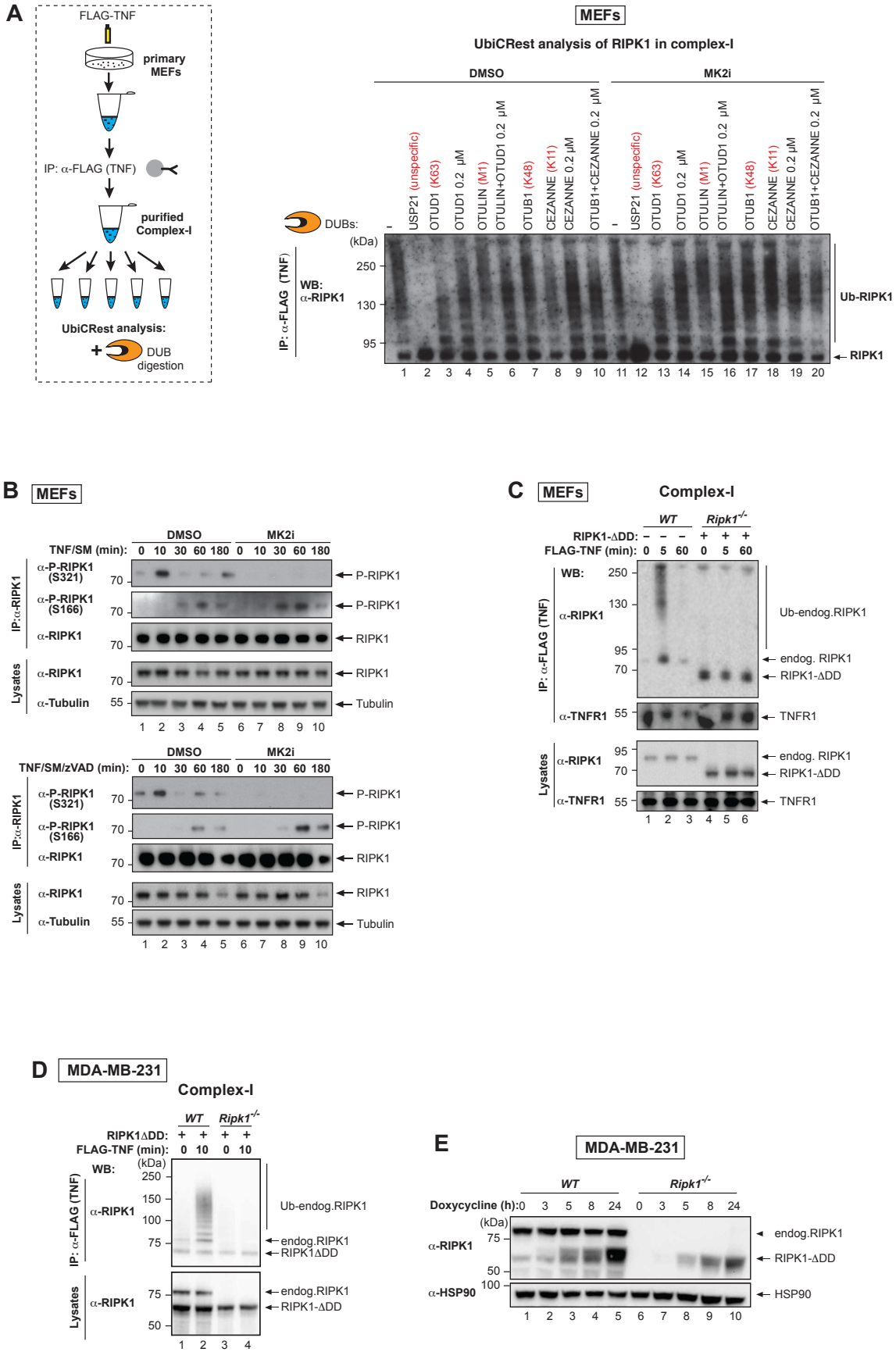
A
FLAG-TNF
primary MEFs
IP: α-FLAG (TNF)
purified Complex-I
UbiCRest analysis:
DUB digestion
MEFs
UbiCRest analysis of RIPK1 in complex-I
DMSO
MK2i
DUBs:
USP21 (unspecific)
OTUD1 (K63)
OTUD1 0.2 μM
OTULIN (M1)
OTULIN+OTUD1 0.2 μM
OTUB1 (K48)
CEZANNE (K11)
CEZANNE 0.2 μM
OTUB1+CEZANNE 0.2 μM
IP: α-FLAG (TNF)
WB: α-RIPK1
(kDa)
250
130
95
Ub-RIPK1
RIPK1
B
MEFs
DMSO
MK2i
TNF/SM (min): 0 10 30 60 180 0 10 30 60 180
IP:α-RIPK1
α-P-RIPK1 (S321)
α-P-RIPK1 (S166)
α-RIPK1
Lysates
α-RIPK1
α-Tubulin
70
55
P-RIPK1
RIPK1
Tubulin
TNF/SM/zVAD (min): 0 10 30 60 180 0 10 30 60 180
C
MEFs
Complex-I
WT
Ripk1-/-
RIPK1-ΔDD: – – – + + +
FLAG-TNF (min): 0 5 60 0 5 60
WB:
IP: α-FLAG (TNF)
α-RIPK1
α-TNFR1
250
130
95
70
55
Ub-endog.RIPK1
endog. RIPK1
RIPK1-ΔDD
TNFR1
Lysates
D
MDA-MB-231
Complex-I
WT
Ripk1-/-
RIPK1ΔDD: + + + +
FLAG-TNF (min): 0 10 0 10
WB:
IP: α-FLAG (TNF)
α-RIPK1
Lysates
(kDa)
250
150
100
75
50
Ub-endog.RIPK1
endog.RIPK1
RIPK1ΔDD
E
MDA-MB-231
WT
Ripk1-/-
Doxycycline (h): 0 3 5 8 24 0 3 5 8 24
α-RIPK1
α-HSP90
(kDa)
75
50
100
endog.RIPK1
RIPK1-ΔDD
HSP90

Figure S4. MK2-dependent phosphorylation of RIPK1 does not affect NF-κB signalling, Related to Figure 4

(A) UbiCRest analysis of ubiquitylated RIPK1 in complex-I. Complex-I was purified from primary WT MEFs using FLAG-mTNF as affinity reagent. Immuno-complexes were then subjected to UbiCRest analysis using the indicated panel of DUBs followed by Western blot analysis for RIPK1. **(B)** Immuno-precipitation of RIPK1 from DMSO and MK2i-treated MEFs (1 μM). The presence of the indicated proteins was evaluated by western blot. Cells were treated with the indicated agents for the indicated time points. **(C,D)** TNF-induced complex-I immuno-precipitation using FLAG-mTNF or FLAG-hTNF (1 μg /ml) as an affinity reagent from lysates of WT and *Ripk1*$^{-/-}$ MEFs (C) and MDA-MB-231 (D) cells stably expressing an inducible form of RIPK1-ΔDD. Western blot analysis with the indicated antibodies is shown. **(E)** Western blot analysis of cell lysates of WT and *Ripk1*$^{-/-}$ MDA-MB-231 cells stably expressing RIPK1-ΔDD. Expression of RIPK1-ΔDD was induced by Doxycycline (1 μg/ml), and the presence and phosphorylation status of the indicated proteins was evaluated.

Figure S5

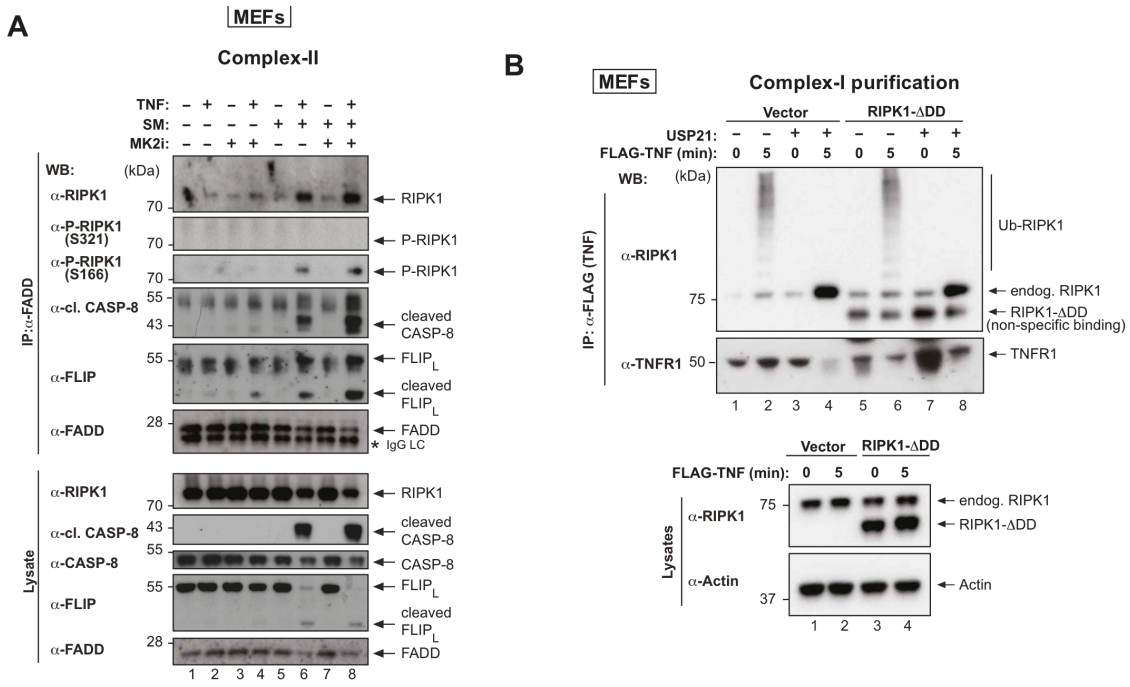

Figure S5. RIPK1-ΔDD is not recruited to TNF complex-I, Related to Figure 5

(A) TNF-induced complex-II was immuno-precipitated with anti-FADD from MEF lysates. Cells were treated with TSZ for 3 hrs ± MK2i (2 μM). **(B)** TNF-induced complex-I immuno-precipitation using FLAG-mTNF (1 μg/ml) as an affinity reagent from lysates of WT MEFs stably expressing and inducible form of RIPK1-ΔDD. Western blot analysis with the indicated proteins is shown.

Figure S6

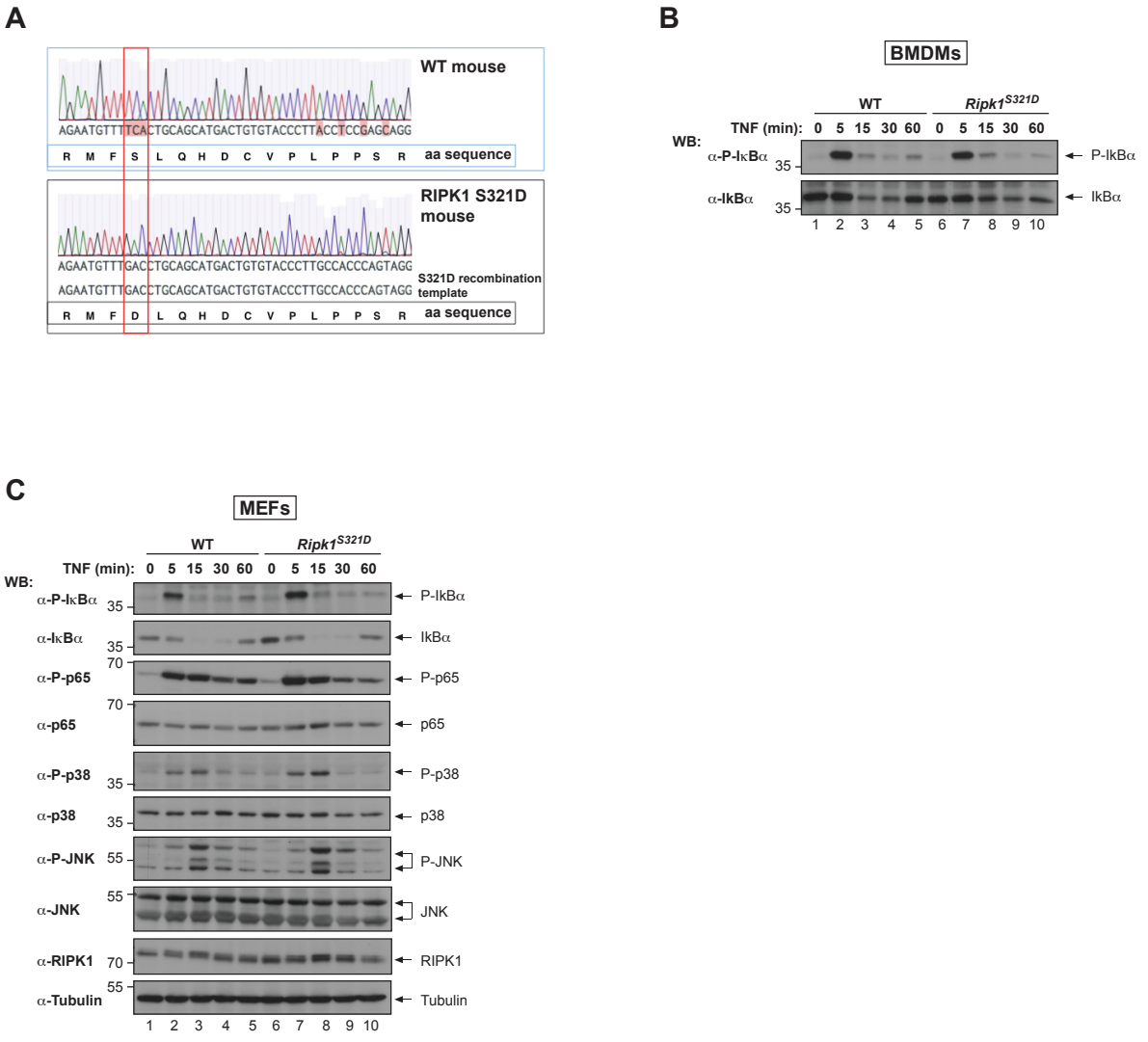

Figure S6. MK2-dependent phosphorylation of RIPK1 at S321 protects cells from TNF induced cell death, Related to Figure 6

(A) Sequence of the *Ripk1* gene in WT and *Ripk1*$^{S321D/S321D}$ mice. In addition to the TCA to GAC mutation changing S at position 321 to D, silent mutations were introduced in the PAM sequence to protect the mutated allele from Cas9-mediated cleavage. **(B, C)** Western blot analysis of primary BMDMs (B) and MEFs (C) isolated from WT and *Ripk1*^{S321D} mice using the indicated antibodies. Cells were treated with TNF (10 ng/ml) for the indicated time points.