Supporting Information

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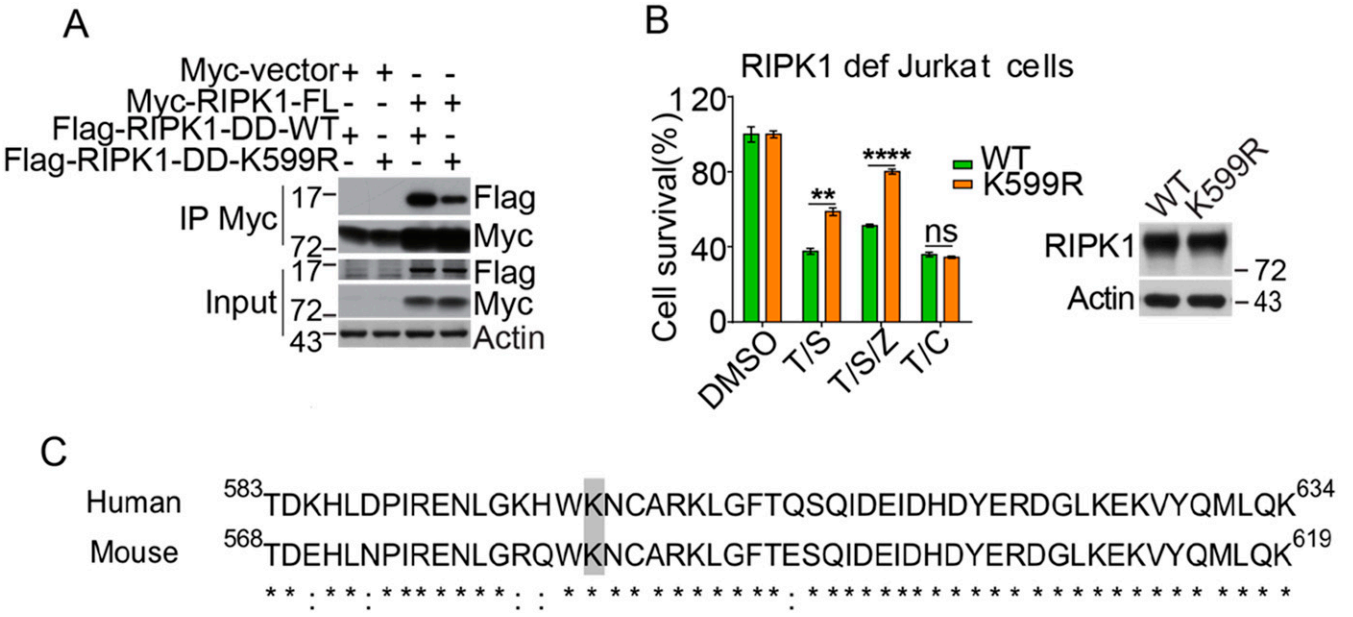

Fig. S1. Effect of hRIPK1 K599R mutation on RIPK1 dimerization and cell death. (*A*) HEK293T cells were cotransfected with expression vectors of Myc-tagged full-length RIPK1, Flag-RIPK1-DD, and Flag-RIPK1-DD-K599R as indicated for 24 h, the cells were then lysed with 0.5% Nonidet P-40 buffer, and cell lysates were immunoprecipitated with anti-Myc antibody-conjugated agarose. (*B*) RIPK1-deficient Jurkat cells were infected with retrovirus encoding Flag-tagged hRIPK1 WT and Flag-tagged hRIPK1 K599R by the Tet-On Advanced Inducible Expression System. Different RIPK1-reconstituted Jurkat cells were treated with 1 μg/mL doxycycline for 48 h to induce the expression of RIPK1. The cells were then pretreated with 100 nM SM-164, 100 nM 5Z-7, or CHX 1 μg/mL for 2 h and treated with 100 ng/mL TNFα and 50 μM zVAD.fmk for 10 h. The cell survival was measured by CellTiterGlo. Results shown are averages of triplicates ± SEM (**$P < 0.01$; ****$P < 0.0001$). (*Right*) Western blot analysis of RIPK1 expression levels in RIPK1-deficient Jurkat cells infected with retrovirus encoding Flag-tagged hRIPK1 WT and K599R. (*C*) Sequence alignment of the death domains of human and murine RIPK1 around the K599/K584 region. K599/K584 residues conserved in humans and mice are highlighted in gray.

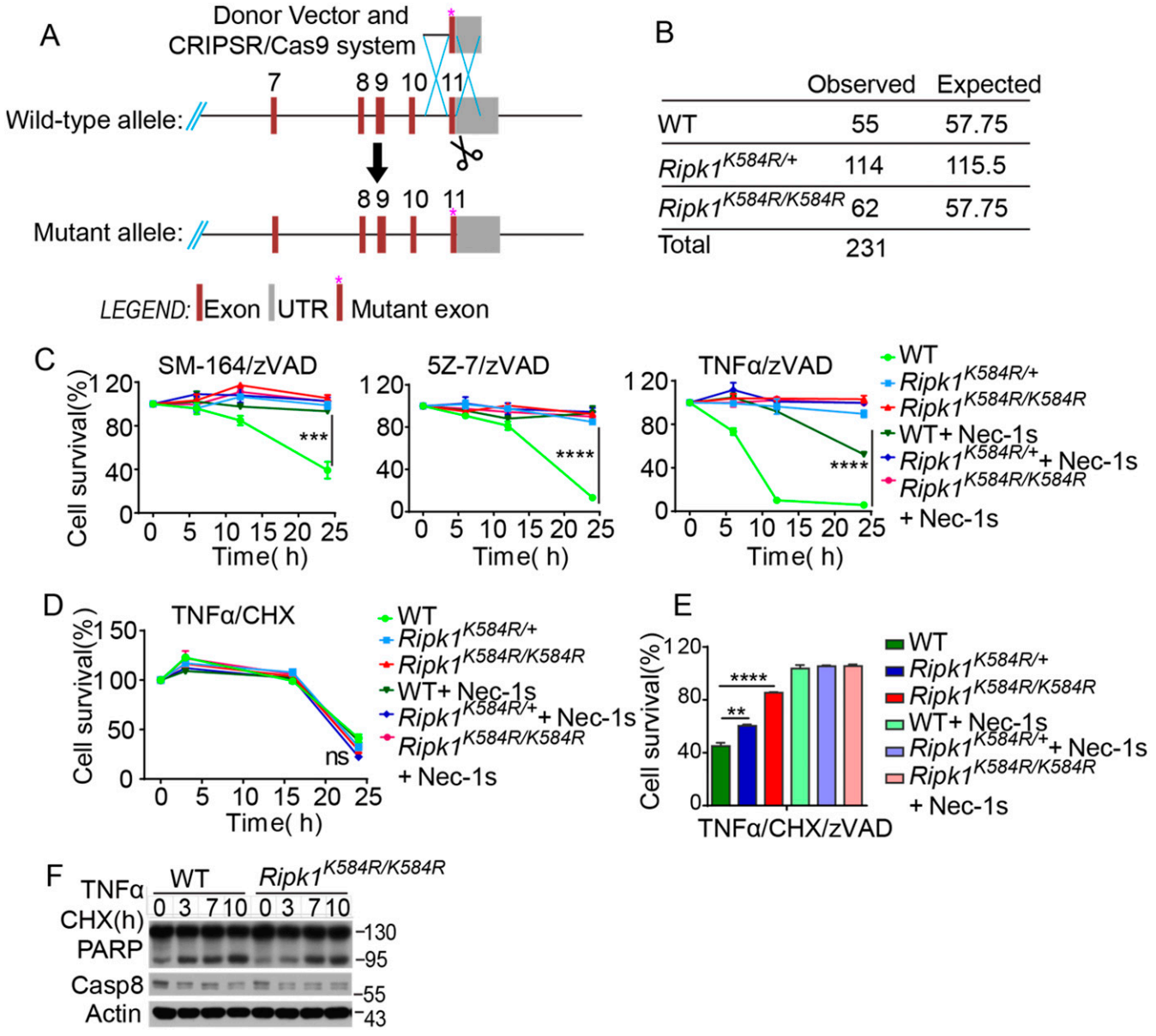

Fig. S2. Ripk1^{K584R} cells are resistant to RIPK1-dependent cell death but not RIPK1-independent apoptosis. (*A*) A schematic diagram of the murine *Ripk1* locus and the *Ripk1* K584R allele. (*B*) Numbers of surviving offspring from mating of *Ripk1*$^{K584R/+}$ parents. (*C*) WT, *Ripk1*$^{K584R/+}$, and *Ripk*$^{K584R/K584R}$ immortalized MEFs were pretreated with 25 nM SM-164 (*Left*) or 100 nM 5Z-7 (*Middle*) for 2 h and then treated with 50 μM zVAD.fmk or treated with 20 ng/mL TNFα and 50 μM zVAD.fmk (*Right*) as indicated for different time points. (*D* and *E*) WT, *Ripk1*$^{K584l/+}$, and *Ripk*$^{K584R/K584R}$ immortalized MEFs were treated with TNFα+CHX to induce apoptosis (*D*) or treated with TNFα+CHX+zVAD.fmk to induce necroptosis (*E*). The cell survival was measured by CellTiterGlo (*A*–*C*). (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.) (*F*) WT and *Ripk*$^{K584R/K584R}$ immortalized MEFs were treated with TNFα+CHX for different time points, and cells were lysed with 0.5% Nonidet P-40 buffer and analyzed by Western blotting with caspase and PARP cleavage to indicate apoptosis.

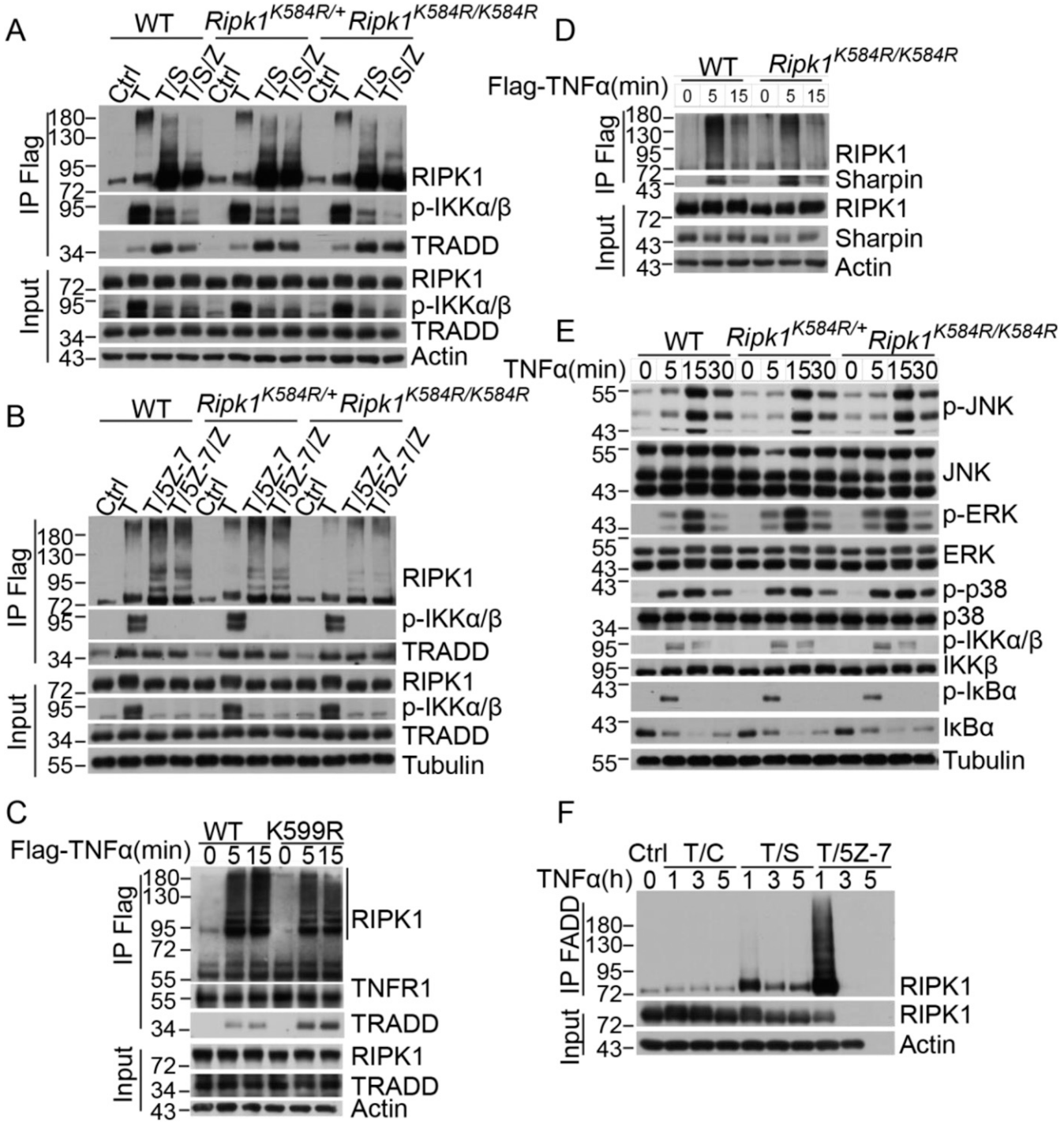

Fig. S3. The *Ripk1*^{K584R} mutation disrupts the formation of complex II. (*A* and *B*) Primary WT, *Ripk1*$^{K584R/+}$, and *Ripk*$^{K584R/K584R}$ MEFs were pretreated with SM-164 ± zVAD (*A*) or 5Z-7 ± zVAD (*B*) for 1 h as indicated, and then Flag-TNFα was added for 5 min, cells were then lysed with 0.5% Nonidet P-40 buffer, and cell lysates were immunoprecipitated with anti-Flag antibody-conjugated agarose. All immunoprecipitated proteins and whole-cell lysates were analyzed by Western blotting with indicated antibodies. (*C*) RIPK1-deficient Jurkat cells infected with retrovirus encoding HA-tagged WT or K599R hRIPK1 were treated with Flag-TNFα for the indicated time. The cells were then lysed with 0.5% Nonidet P-40 buffer and immunoprecipitated with anti-Flag antibody-conjugated agarose. (*D*) Primary WT and *Ripk1*$^{K584R/K584R}$ MEFs were treated with TNFα and then lysed with 0.5% Nonidet P-40 buffer and immunoprecipitated with anti-Flag antibody-conjugated agarose. (*E*) WT, *Ripk1*$^{K584R/+}$, and *Ripk*$^{K584R/K584R}$ primary MEFs were treated with 50 ng/mL TNFα at various time points. The cell lysates were analyzed by phosphorylated and total JNK, ERK, p38, IKKα/β, IκBα, and actin as indicated. (*F*) Immortalized MEFs were pretreated with CHX, SM-164, or 5Z-7 for 2 h and then treated with TNFα as indicated at different time points. The cells were lysed with 0.5% Nonidet P-40 buffer and immunoprecipitated with anti-FADD antibody. The immunoprecipitated complexes and whole-cell lysates were analyzed by Western blotting with RIPK1.

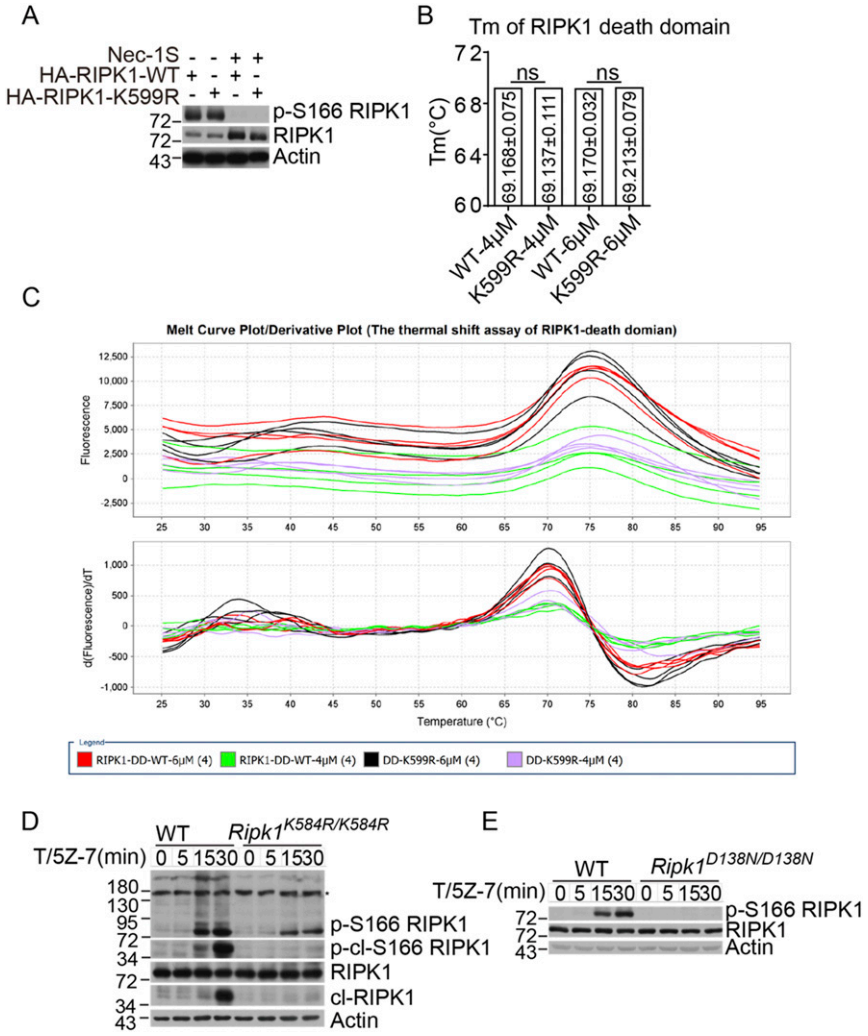

Fig. S4. The RIPK1 K584R mutation does not directly inhibit RIPK1 kinase activity or affect protein stability. (*A*) The RIPK1 KO 293T cells were transfected with HA-tagged RIPK1 WT or K599R expression plasmids in the presence or absence of 10 μM Nec-1s. The cell lysates were collected 24 h after transfection and analyzed by Western blotting with the indicated antibodies. (*B* and *C*) The melting temperature (Tm) (*B*) and thermal stability profiles (*C*) of WT and K599R hRIPK1-DD. hRIPK1 (583-671AA) was purified from the *E. coli* expression system following overnight induction at 4 °C. hRIPK1-DD WT and K599R (4 μM, 6 μM) protein thermal stability were analyzed through the use of differential scanning calorimetry by real-time PCR and the melting temperatures were calculated by Protein Thermal Shift Software. Four replicates for each reaction were performed. (*D* and *E*) WT and *Ripk1*$^{K584R/K584R}$ (*D*) and WT *and Ripk1*$^{D138N/D138N}$ (*E*) immortalized MEFs were pretreated with 5Z-7 for 2 h and then treated with TNFα for the indicated time. The cells were lysed with 0.5% Nonidet P-40 buffer and analyzed by Western blotting analysis using p-S166 RIPK1 and RIPK1 antibodies.