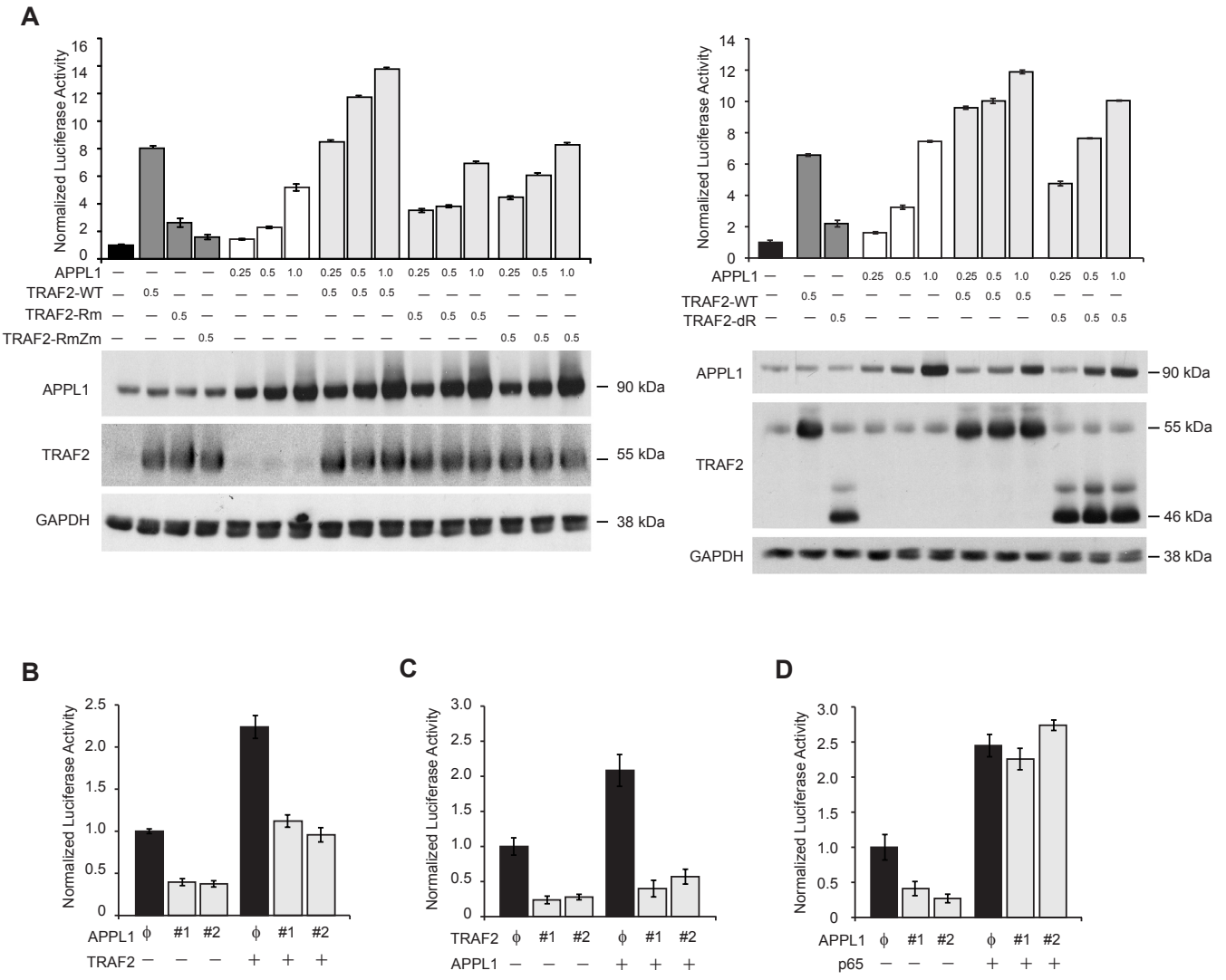

Fig. S1 APPL1 and TRAF2 synergize in the activation of the NF-κB pathway. (A) E3 ligase-deficient TRAF2 mutants synergize with APPL1 in the NF-κB activation. HEK293T cells were cotransfected with reporter vectors together with the different combinations of APPL1, TRAF2-WT, TRAF2-Rm, TRAF2-RmZm or TRAF2-dR expression vectors, as indicated (all values are in μg of DNA). The luciferase activity was measured as described in Materials and Methods. APPL1 and TRAF2 levels were confirmed by immunoblotting (lower panels) with GAPDH level as a loading control. **(B)** APPL1 is necessary for the TRAF2-mediated NF-κB reporter activity. HEK293T cells were cotransfected with reporter vectors together with APPL1 siRNA #1 and #2 or non-targeting siRNA (ϕ) alone or in combination with TRAF2 expression vector. (**C)** TRAF2 is necessary for the APPL1-mediated NF-κB activity. HEK293T cells were cotransfected with reporter vectors and TRAF2 esiRNA #1 and #2 or control esiRNA (ϕ) either alone or in combination with APPL1. **(D)** p65 overexpression overcomes an inhibitory effect of APPL1 depletion on the NF-κB reporter. HEK293T cells were cotransfected with reporter vectors together with APPL1 siRNA #1 and #2 or non-targeting siRNA (ϕ) alone or in combination with p65 expression vector.

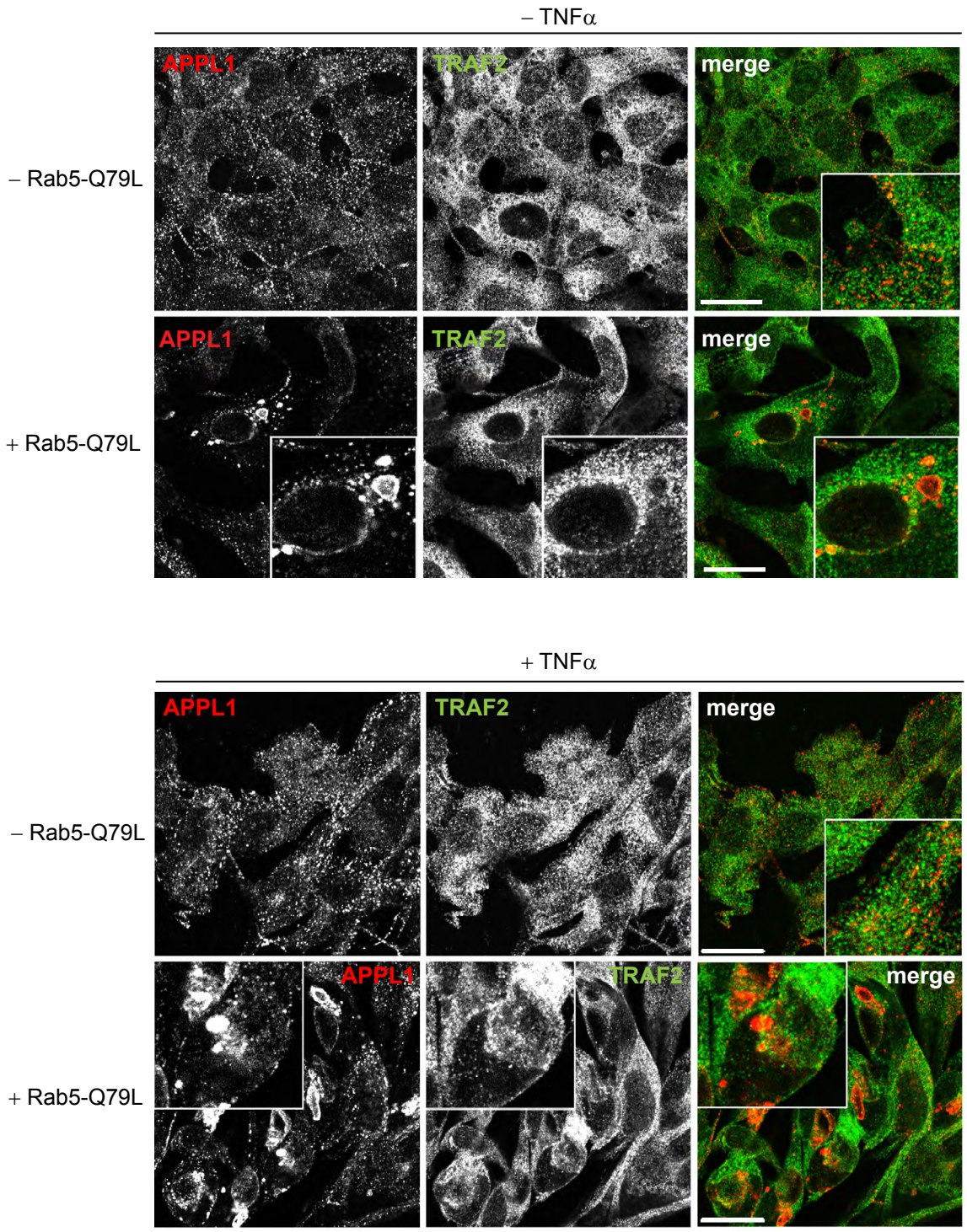

Fig. S2 TRAF2 is not detectable on endosomes. Twenty-four h after transfection with Rab5-Q79L, HEK293T cells were stimulated with TNFα for 10 min or left untreated, fixed and stained with antibodies against APPL1 (red) and TRAF2 (green). Scale bar, 20 µm.

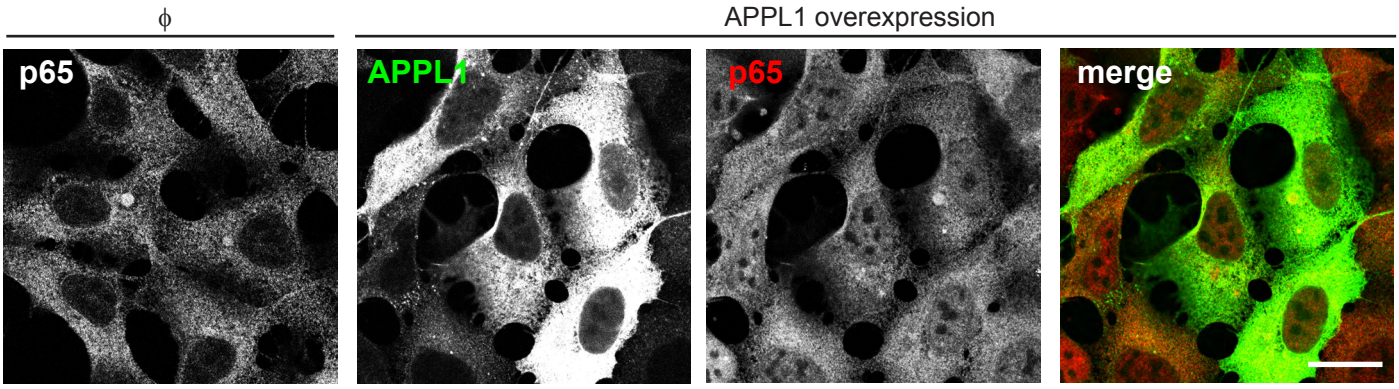

Fig. S3 APPL1 overexpression enhances nuclear localization of p65 in HEK293T cells. Twenty-four h after transfection with HA-APPL1, HEK293T cells were fixed and stained with anti-p65 (red) and anti-HA (APPL1, green) antibodies. Scale bar, 20 μm.

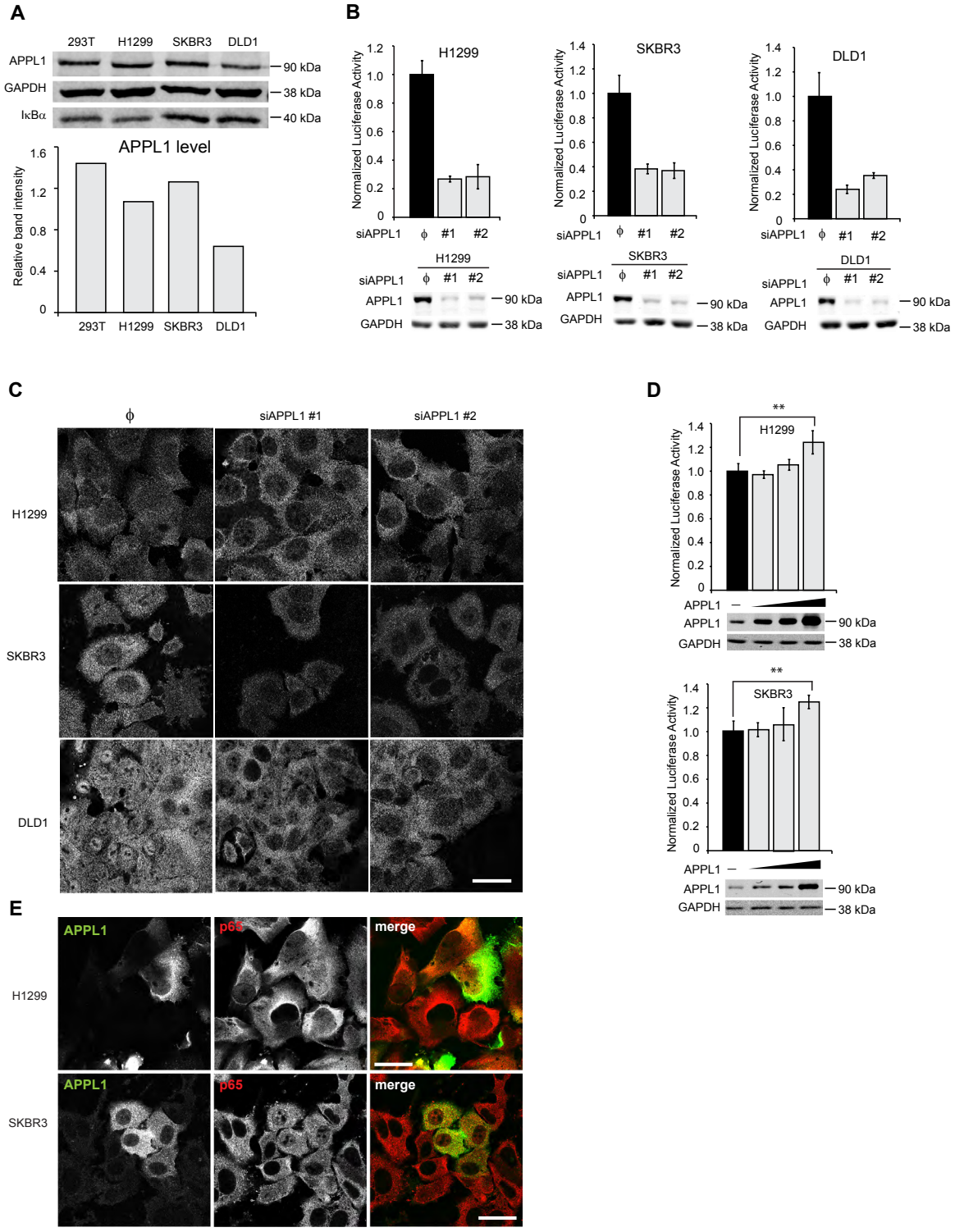

Fig. S4 APPL1 modulates the NF-κB activity in cancer cells. (A) Endogenous levels of APPL1 in cancer cells. The levels of APPL1, IκBα and GAPDH (loading control) in cell lysates from HEK293T, H1299, SKBR3 and DLD1 cells were analyzed by Western blotting. Band intensity of APPL1 was quantified compared to GAPDH (lower panel). A representative result from four independent experiments is shown. **(B)** APPL1 knockdown inhibits the NF-κB reporter in cancer cells. H1299, SKBR3 and DLD1 cells were cotransfected with reporter plasmids along with APPL1 siRNA #1 and #2 or non-targeting siRNA (ϕ). The luciferase activity was measured as described in Materials and Methods. Knockdown of APPL1 level was confirmed by immunoblotting (lower panels) with GAPDH level as a loading control. **(C)** APPL1 knockdown inhibits p65 translocation to the nucleus in cancer cells. H1299, SKBR3 and DLD1 cells transfected with non-targeting siRNA (ϕ) or APPL1 siRNAs (#1 or #2) were fixed after 48 h and stained with anti-p65 antibody (scale bar, 20 μm). **(D)** High amounts of overexpressed APPL1 activate the NF-κB reporter in cancer cells. H1299 and SKBR3 cells were cotransfected for 48 h with reporter plasmids along with 0.25, 0.5 and 1 μg of APPL1 expression vector and the luciferase activity was measured. The p-values were calculated upon comparison of overexpression of APPL1 to the overexpression of an empty vector and were marked with the asterisks (**p<0.005). **(E)** APPL1 overexpression enhances nuclear localization of p65 in cancer cells. Twenty-four h after transfection with HA-APPL1, H1299 and SKBR3 cells were fixed and stained with anti-p65 (red) and anti-HA (APPL1, green) antibodies. Scale bar, 20 μm.

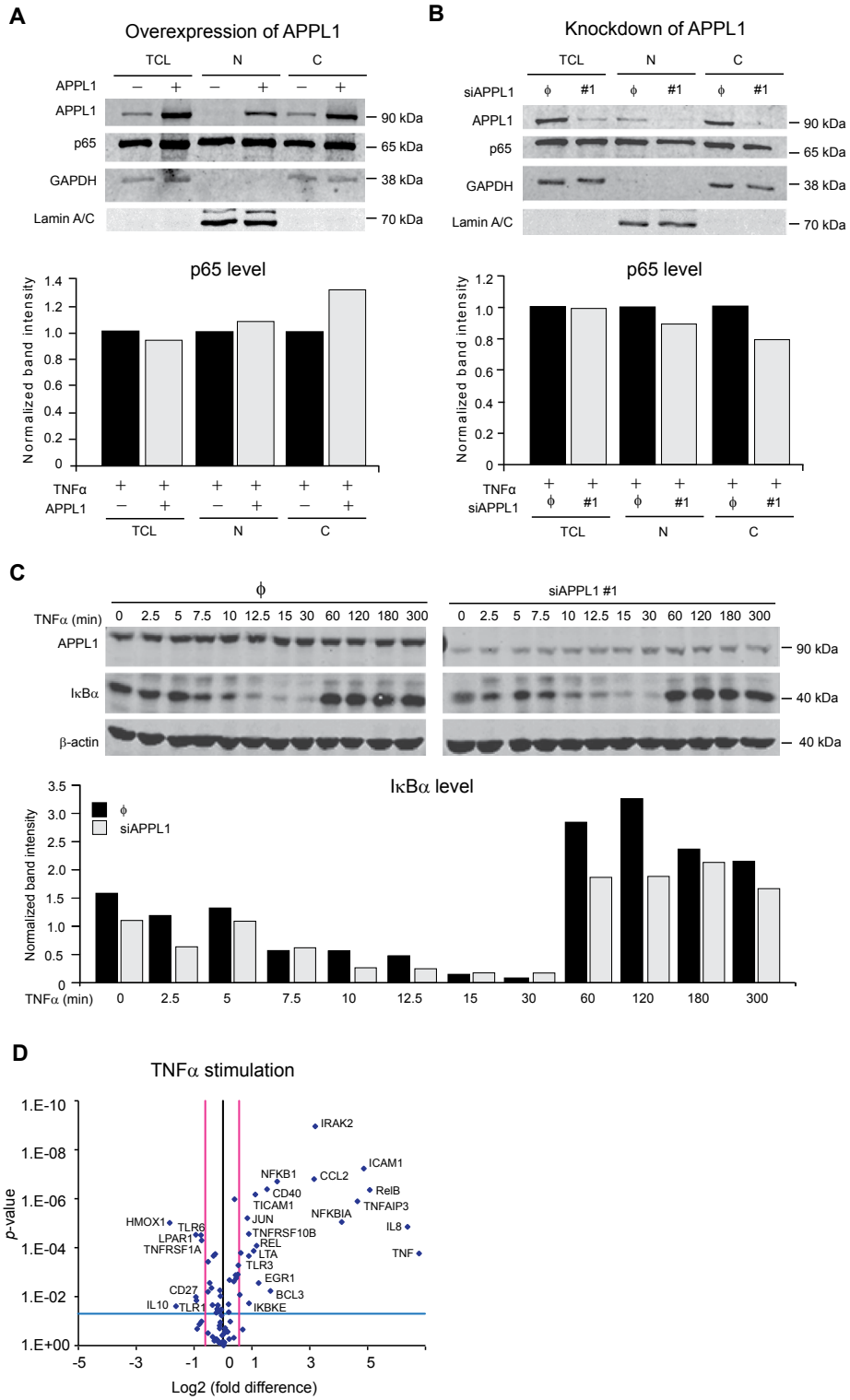

Fig. S5 APPL1 is not necessary for TNFα-mediated NF-κB activation. (A) APPL1 overexpression does not enhance nuclear localization of p65 upon TNFα stimulation. HEK293T cells were transfected with APPL1-expressing vector or an empty vector as a control. Forty-eight h after transfection cells were treated with TNFα for 30 min. Cell fractions were analyzed by Western blotting with anti-APPL1, p65, GAPDH and Lamin A/C antibodies (upper panel). Band intensity of p65 was quantified (lower panel). **(B)** APPL1 knockdown does not affect nuclear localization of p65 upon TNFα stimulation. HEK293T cells were transfected for 48 h with APPL1 siRNA #1 or non-targeting siRNA (ϕ) followed by treatment with TNFα for 30 min. Cell fractions were analyzed as in (A). **(C)** APPL1 knockdown does not affect IκBα degradation. HEK293T cells were transfected with APPL1 siRNA #1 or non-targeting siRNA (ϕ). Lysates from cells stimulated with TNFα for the indicated time points were analyzed for IκBα, APPL1 and β-actin levels (upper panel). Both control and APPL1-depleted samples were run on the same gel, which was cut for the transfer. The band intensity was determined and shown as ratio of IκBα to β-actin (lower panel). **A-(C)** Representative results from three independent experiments are shown. **(D)** Analysis of NF-κB-dependent gene expression upon TNFα stimulation in HEK293T cells. A predesigned 96-well array containing primer pairs for detection of 84 genes involved in NF-κB signaling was used. The vertical lines indicate the 1.5-fold change in gene expression threshold. The horizontal line indicates the p-value=0.05 of the t-test threshold. Only genes with fold changes <-1.5 or >1.5 were considered as significantly down- or upregulated and their names are mentioned on the charts.

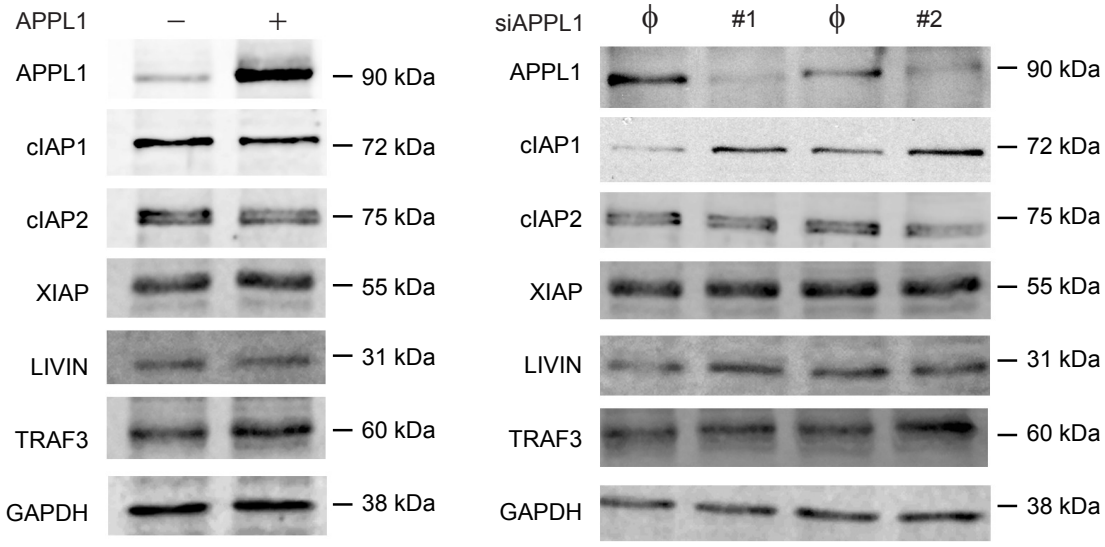

Fig. S6 Effects of APPL1 on the total protein levels of IAP E3 ligases. HEK293T cells were transfected with APPL1 expression vector (left panel) or with APPL1 siRNA #1 and #2 or non-targeting siRNA (ϕ) (right panel). Forty-eight h after transfection, the levels of APPL1, cIAP1, cIAP2, XIAP, livin, TRAF3 and GAPDH (loading control) were analyzed by Western blotting.