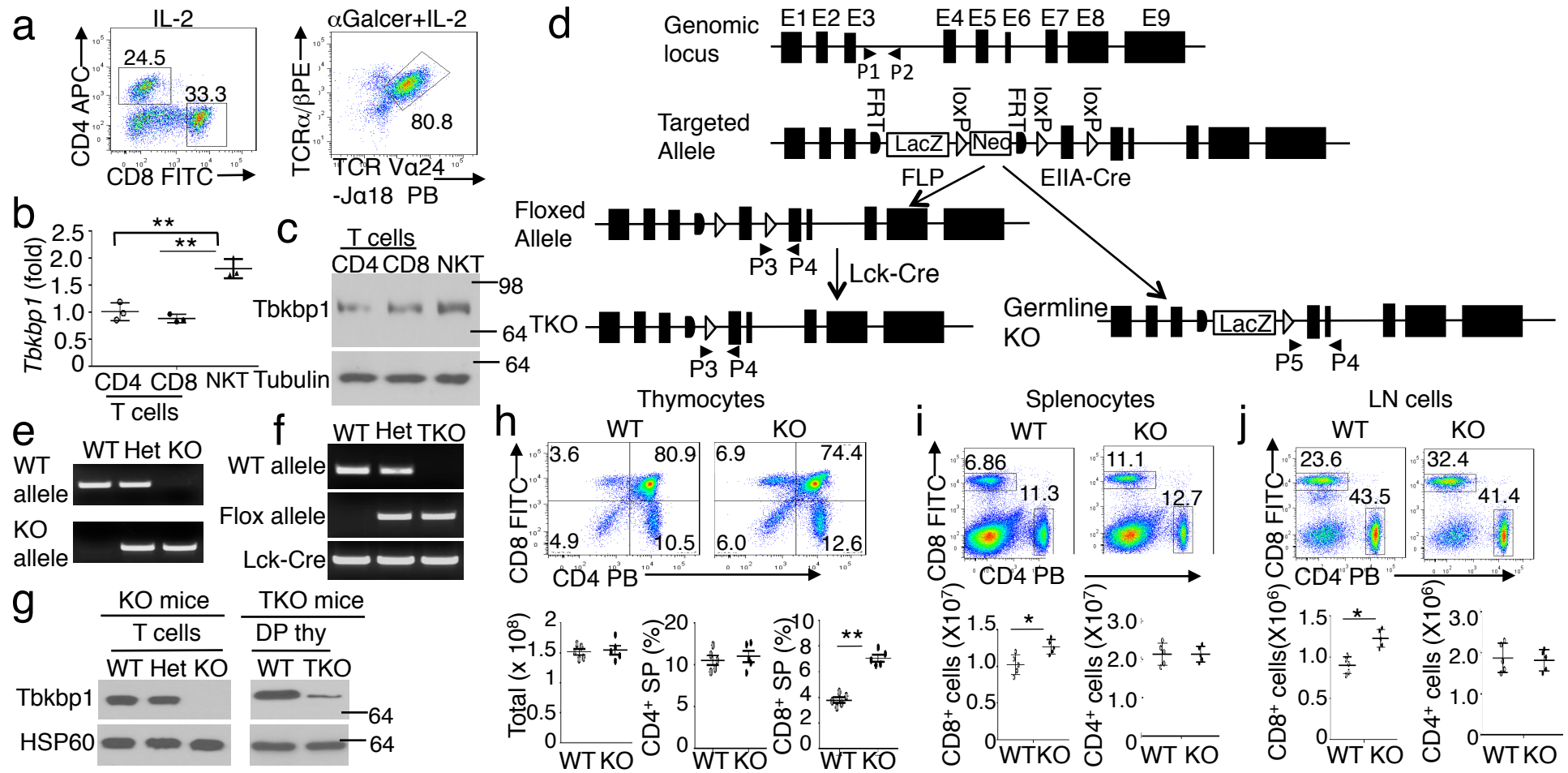


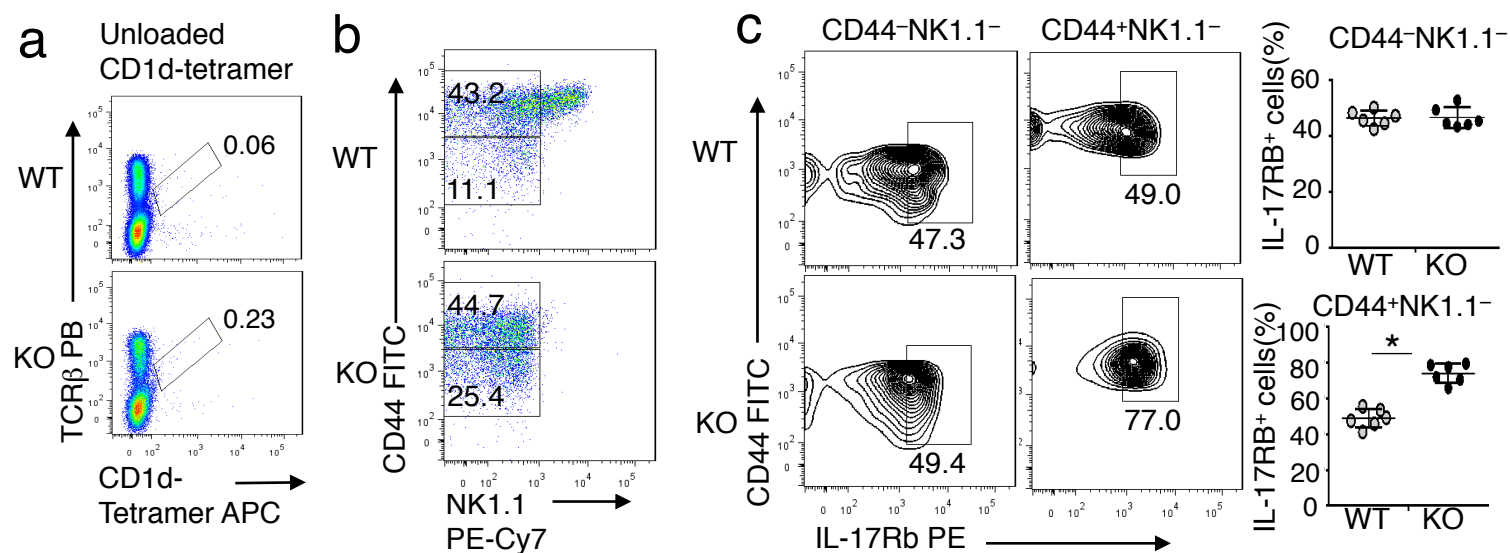
TBK-binding protein 1 regulates IL-15-induced autophagy and NKT cell survival

Zhu et al

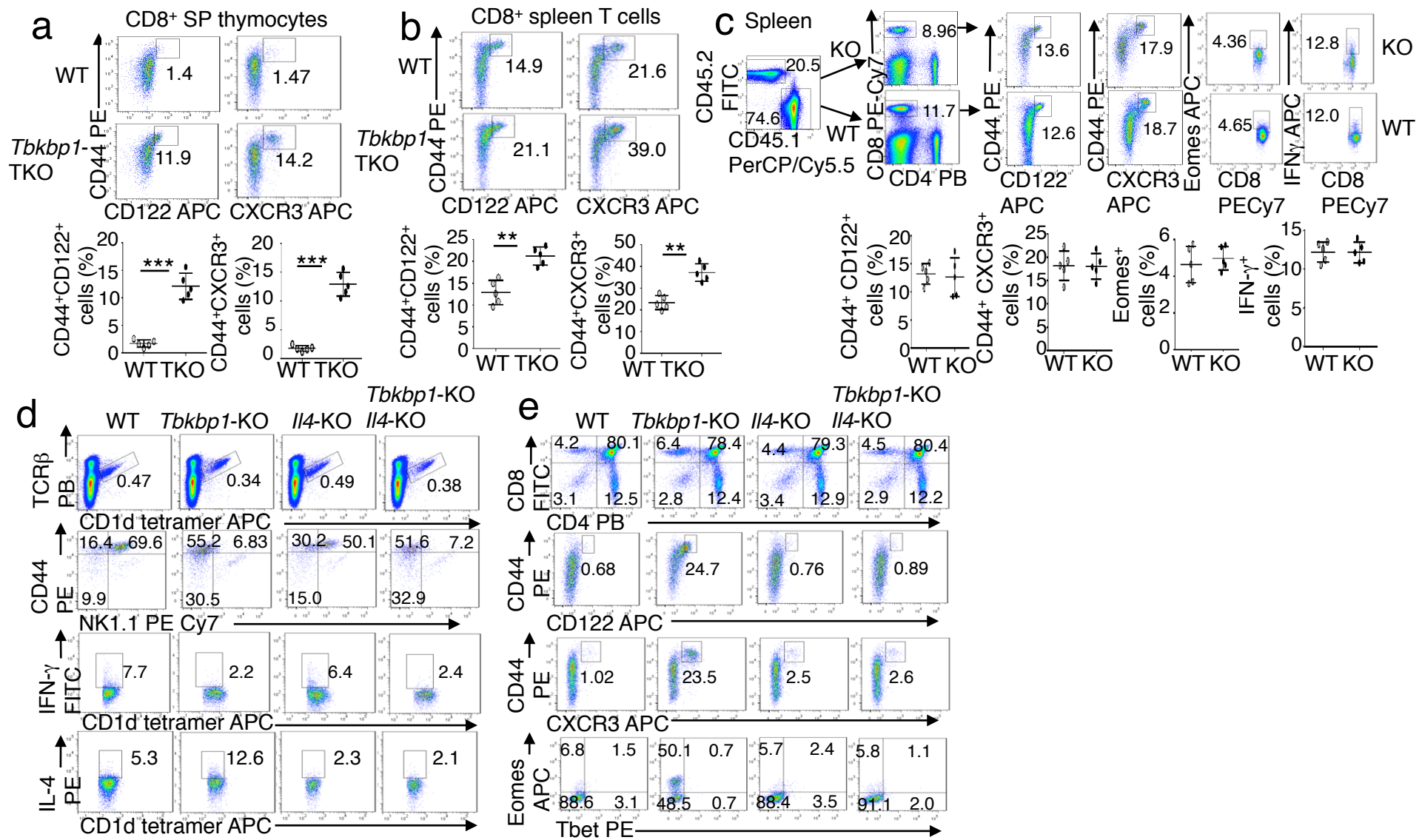
Supplementary Figures 1-14
Supplementary Table 1



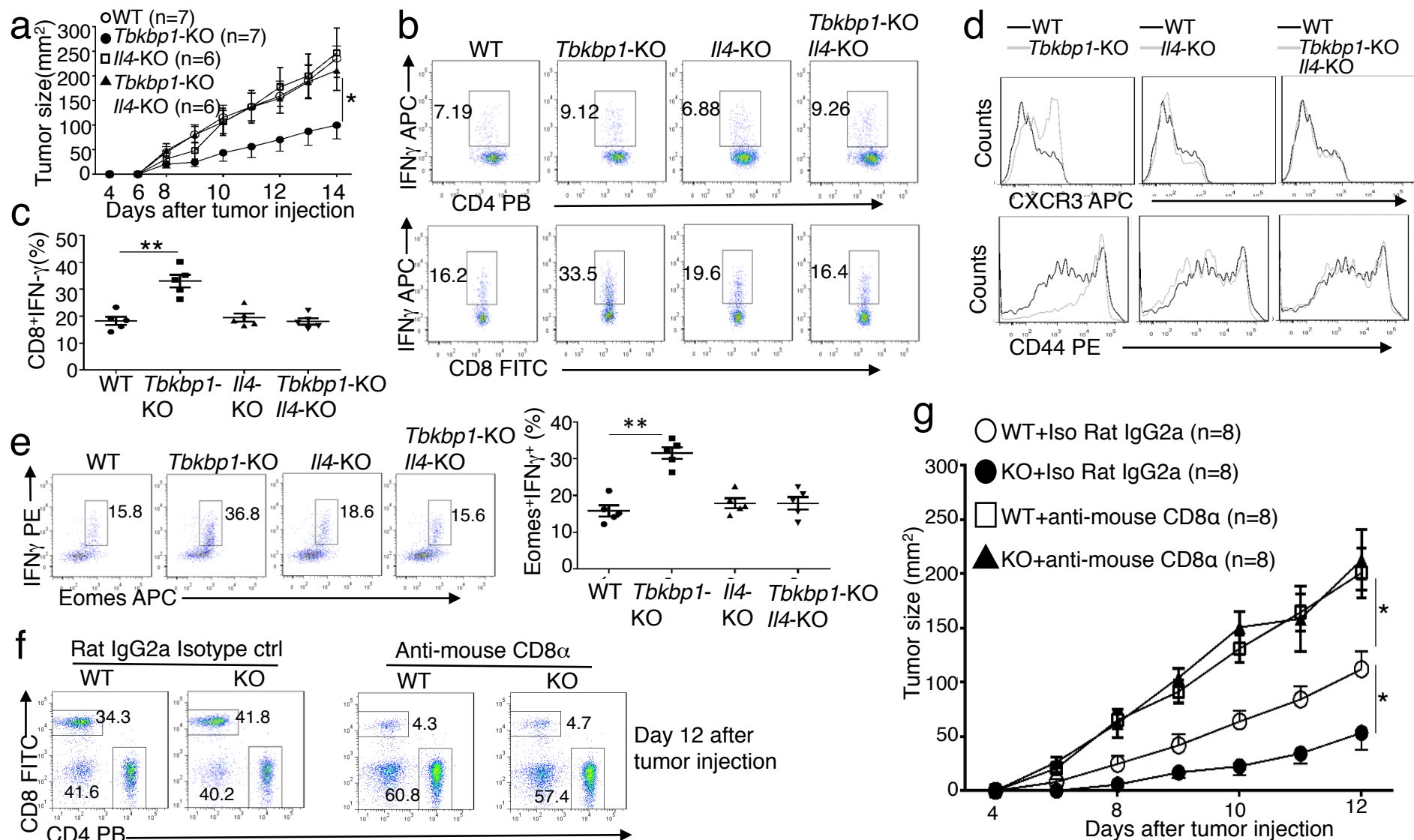
Supplementary Fig. 1. *Tbkbp1* deletion has no effect on thymocyte development. **a**, Flow cytometric analysis of T cells (left) and NKT cells (right) in human peripheral blood mononuclear cells (PBMCs) cultured *in vitro* for 7 days in the presence of IL-2 or for 14 days in the presence α -Galcer plus IL-2. **b,c**, qRT-PCR (**b**) and IB (**c**) analysis of *Tbkbp1* mRNA and protein in CD4⁺ and CD8⁺ T cells or NKT cells sorted from the PBMCs described in **a**. **d**, Schematic picture of *Tbkbp1* gene targeting. Mice carrying the *Tbkbp1* targeted allele (*Tbkbp1*^{tm1a(EUCOMM)Wtsi}) were crossed with EIIA-Cre to generate germline *Tbkbp1*-knockout (KO) mice and with FLP deleter (*Rosa26-FLPe*) mice to generate *Tbkbp1*-floxed mice, which were further crossed with *Lck-Cre* to generate T cell-conditional KO (TKO) mice. **e**, Genotyping PCR analysis of germline *Tbkbp1* wildtype (WT), heterozygous (Het), and KO mice using P1/P2 and P4/P5 primer pairs for WT and KO alleles, respectively. **f**, Genotyping PCR analysis of *Tbkbp1* WT (*Tbkbp1*^{+/+}Lck-Cre), TKO Het (*Tbkbp1*^{+/fl}Lck-Cre), and TKO (*Tbkbp1*^{fl/fl}Lck-Cre) mice using P1/P2 and P3/P4 primer pairs for WT and Flox alleles, respectively. **g**, IB analysis of *Tbkbp1* and control HSP60 proteins in spleen T cells of *Tbkbp1*-KO and control (WT and heterozygous) mice and CD4⁺CD8⁺ double-positive (DP) thymocytes of *Tbkbp1*-TKO and WT control mice. **h**, Flow cytometric analysis of thymocyte subpopulations showing a representative FACS plot (upper) and summary graph (lower) of total thymocyte numbers and frequency of CD4⁺ and CD8⁺ single positive (SP) thymocytes. **i,j**, Flow cytometric analysis of peripheral T cells in the spleen (**i**) and inguinal lymph nodes (iLN) (**j**), showing representative FACS plots (upper) and summary graphs (lower). *P<0.05; **P<0.01. One-way ANOVA (**b**), Student's t-test (**h,i,j**).



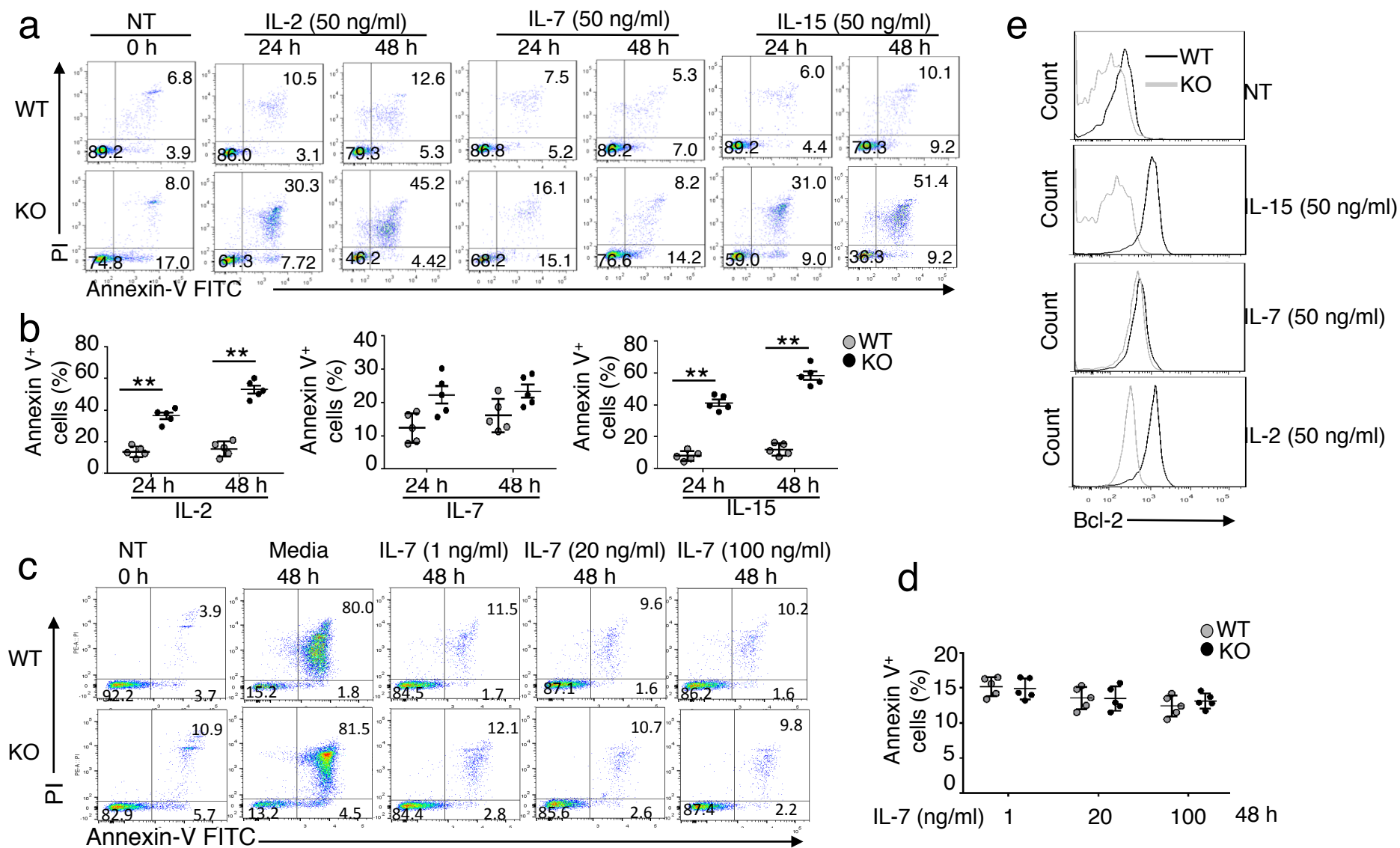
Supplementary Fig. 2. *Tbkbp1* deficiency increases the frequency of IL-17Rb⁺ NKT cells in the CD44⁺NK1.1⁻ subpopulation. **a**, Negative control of flow cytometric analysis of liver NKT cells using an unloaded CD1d-tetramer. **b,c**, Flow cytometric analysis of NKT cells based on CD44 and NK1.1 markers (**b**) and expression of IL-17Rb on gated thymic CD44⁻NK1.1⁻ and CD44⁺NK1.1⁻ NKT subpopulations, presented as representative plots and summary graphs based on multiple mice (each circle represents a mouse) (**c**). *P<0.05. Student's t-test (**c**).



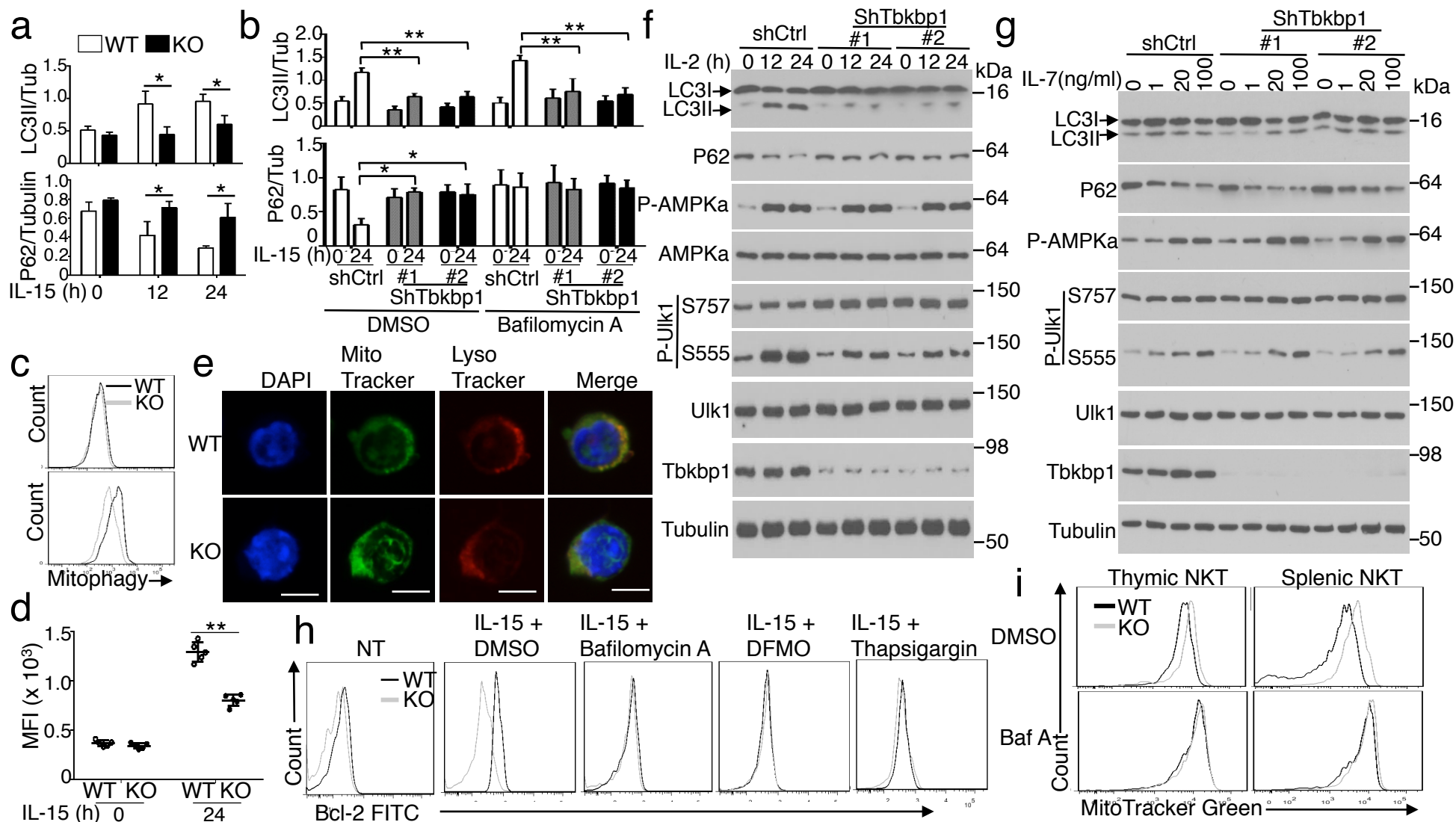
Supplementary Fig. 3. Cell-extrinsic and IL-4-dependent role of *Tbkbp1* in regulating memory-like CD8⁺ T cell generation. **a,b**, Flow cytometric analysis of memory-like CD8⁺ T cells based on CD44^{hi}CD122⁺ and CD44^{hi}CXCR3⁺ surface markers in thymocytes (**a**) and splenocytes (**b**), presented as representative plots (upper) and summary graphs (lower). **c**, Flow cytometric analysis of memory-like CD8⁺ T cells based on expression of CD44, CD122, CXCR3, Eomes and IFN-γ in spleen CD8⁺ T cells of Rag1-KO recipient mice adoptively transferred (for 6 wk) with a mixture of BM cells derived from WT B6.SJL mice (CD45.1⁺) and *Tbkbp1*-KO mice (CD45.2⁺), gating on CD45.1⁺ (WT) or CD45.2⁺ (KO) cells, presented as a representative FACS plot (upper) and summary graphs based on 5 chimeric mice (lower). **d,e**, Flow cytometric analysis of NKT cell maturation and cytokine production (**d**) and memory-like CD8⁺ T cells (**e**) in the thymocytes of the indicated mouse strains. Data are representative of three independent experiments and are presented as means ± s.d. values. **P<0.01; ***P<0.001. Student's t-test (**a,b,c**).



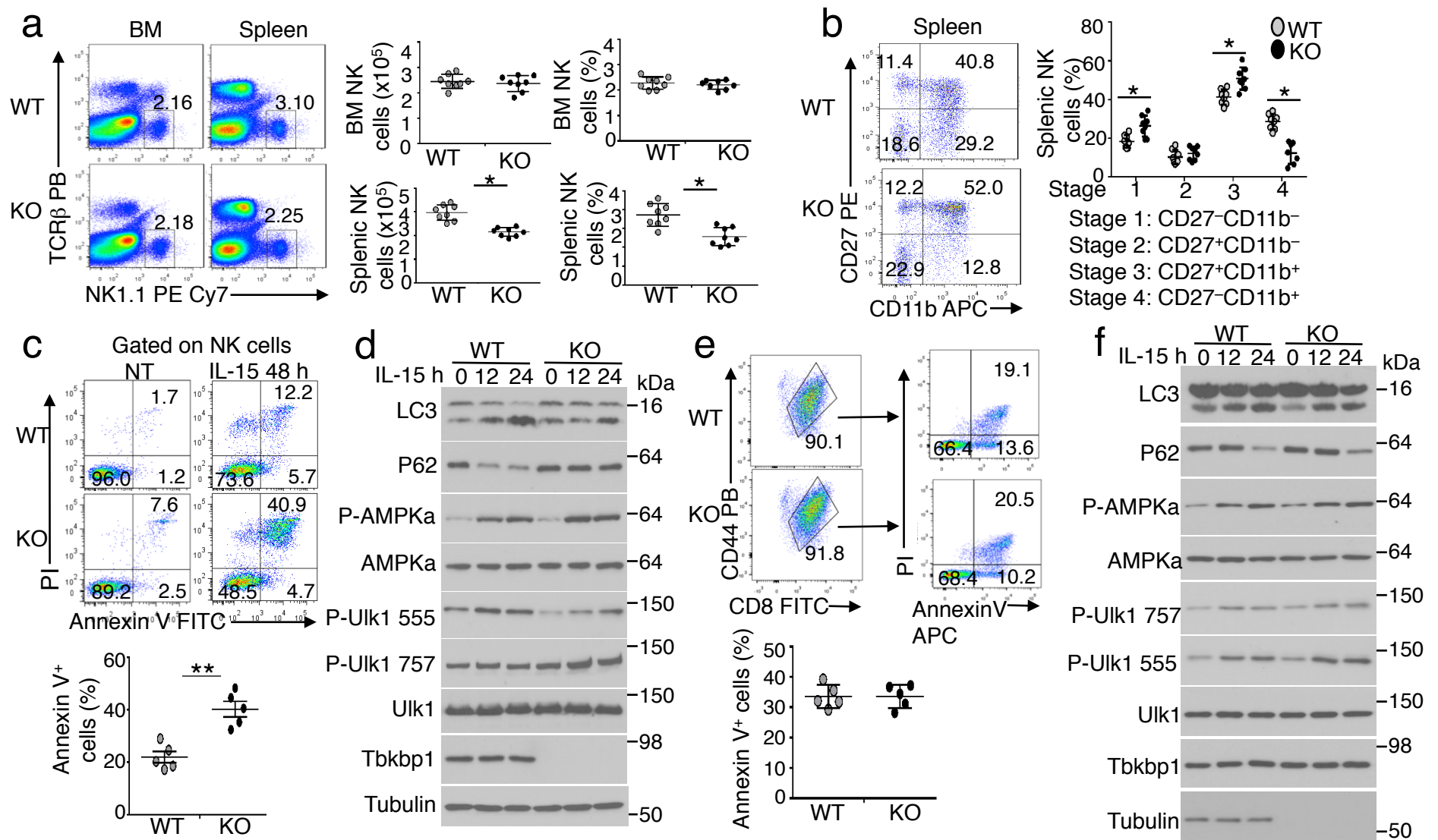
Supplementary Fig. 4. *Tbkbp1* regulates CD8 T cell-dependent antitumor immunity. **a-c**, Tumor growth curve (**a**) and flow cytometric analysis of IFN γ -producing CD4⁺ and CD8⁺ effector T cells in day 14 tumors (**b**) of the indicated mice injected with B16-OVA melanoma cells, presented as representative graphs (**b**) and a summary graph (**c**). **d**, Flow cytometric analysis of expression of CXCR3 or CD44 on gated CD8⁺ T cells in day14 tumors of the indicated mice injected with B16-OVA. **e**, Flow cytometric analysis of IFN γ ⁺Eomes⁺ cells in tumor-infiltrating CD8⁺ T cells of the indicated mice injected with B16-OVA (day 14), presented as representative plots (left) and a summary graph (right). **f,g**, Flow cytometric analysis of draining lymph node cells for monitoring CD8⁺ T-cell depletion (**f**) and tumor growth curve (**g**) of WT and *Tbkbp1*-KO mice injected with B16-OVA along with a CD8 α neutralizing antibody or an IgG2a isotype control. *P<0.05; **P<0.01. Two-way ANOVA (**a,g**), One-way ANOVA (**c,e**)



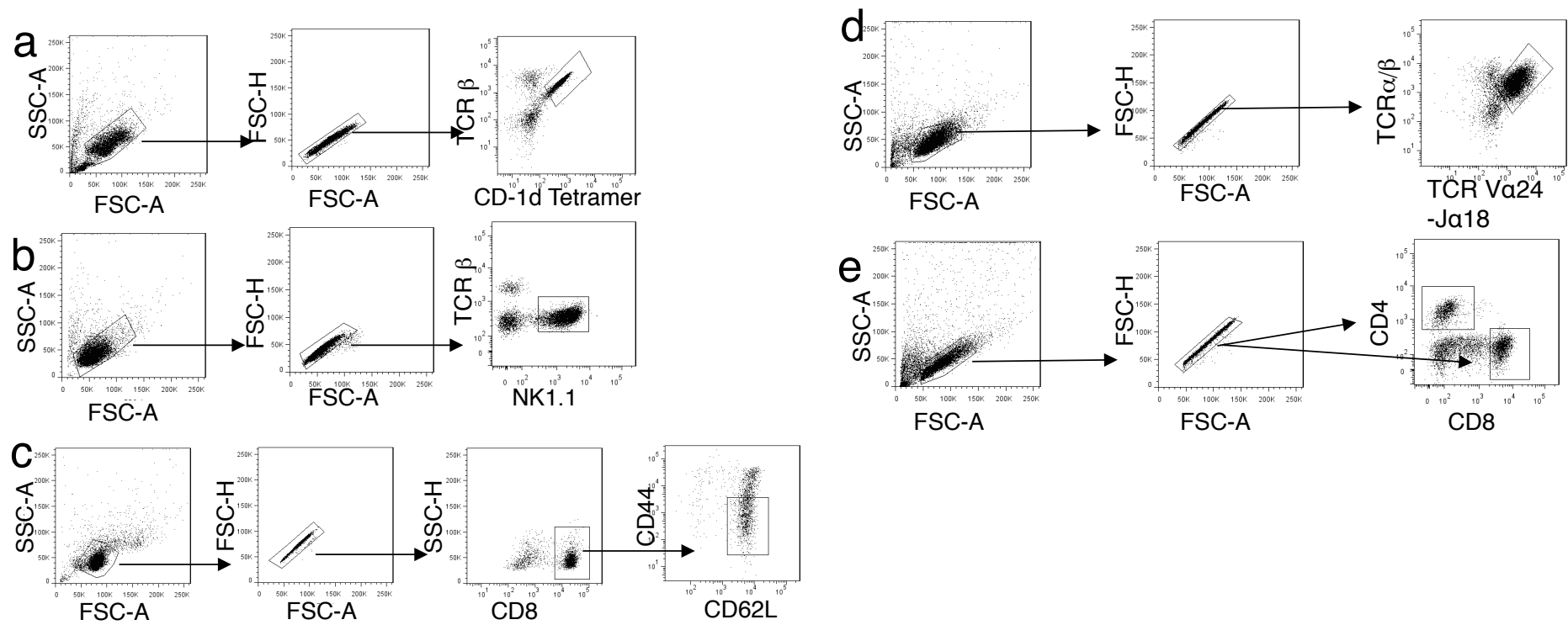
Supplementary Fig. 5. *Tbkbp1* is required for NKT cell survival mediated by IL-15 and IL-2 but not by IL-7. a-d, Flow cytometric apoptosis analysis of WT or *Tbkbp1*-KO (KO) NKT cells that were either not treated (NT) or incubated with the indicated cytokines for the indicated times. Data are presented as a representative plot (**a**, **c**) and summary graphs based on multiple mice (each circle represents a mouse) (**b**, **d**). **e**, Flow cytometric analysis of intracellular Bcl2 expression level in WT or *Tbkbp1*-KO (KO) NKT cells incubated with the indicated cytokines for 48 h. Data are representative of three independent experiments and are presented as means $\pm$ SD. **, $P < 0.01$. Student's t-test (**b**, **d**).



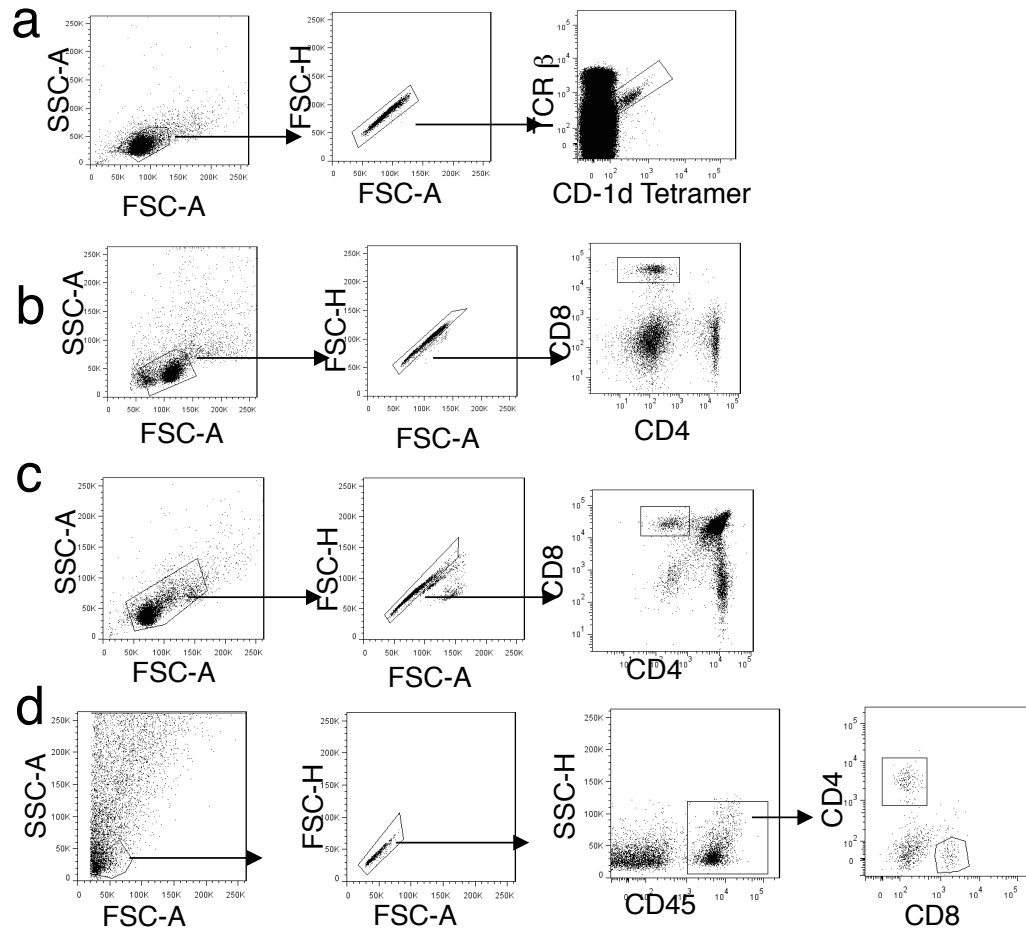
Supplementary Fig. 6. TBKBP1 is required for NKT cell autophagy induced by IL-2 and IL-15 but not by IL-7. a,b, Densitometry quantification of Fig. 6a (a) and Fig. 6c (b). **c,d**, Mitophagy analysis by flow cytometry, presented as a representative plot (c) and summary graph (d). **e**, Confocal imaging analysis of freshly isolated WT or *Tbkbp1*-KO (KO) NKT cells, showing mitochondria-lysosome colocalization in WT but not KO NKT cells. Scale bar, 5 μm. **f,g**, IB analyses of the indicated phosphorylated (P-) and total proteins in whole-cell lysates of NKT hybridoma cells (NKT1.2) stably transduced with a control shRNA or two different *Tbkbp1* shRNAs following stimulation with IL-2 (50 ng/ml) for the indicated times (f) or with the indicated doses of IL-7 for 24 h (g). **h**, Flow cytometric analysis of Bcl-2 expression in WT and *Tbkbp1*-KO (KO) enriched thymic NKT cells that were either not treated (NT) or incubated for 48 h with IL-15 in the presence of DMSO or the indicated autophagy inhibitors. **i**, Flow cytometric analysis of mitochondrial mass in WT or *Tbkbp1*-KO (KO) thymic or splenic NKT cells incubated for 12 h with IL-15 in the presence of DMSO or bafilomycin A (Baf A). *P<0.05; **P<0.01. Two-way ANOVA (a,b), Student's t-test (d).



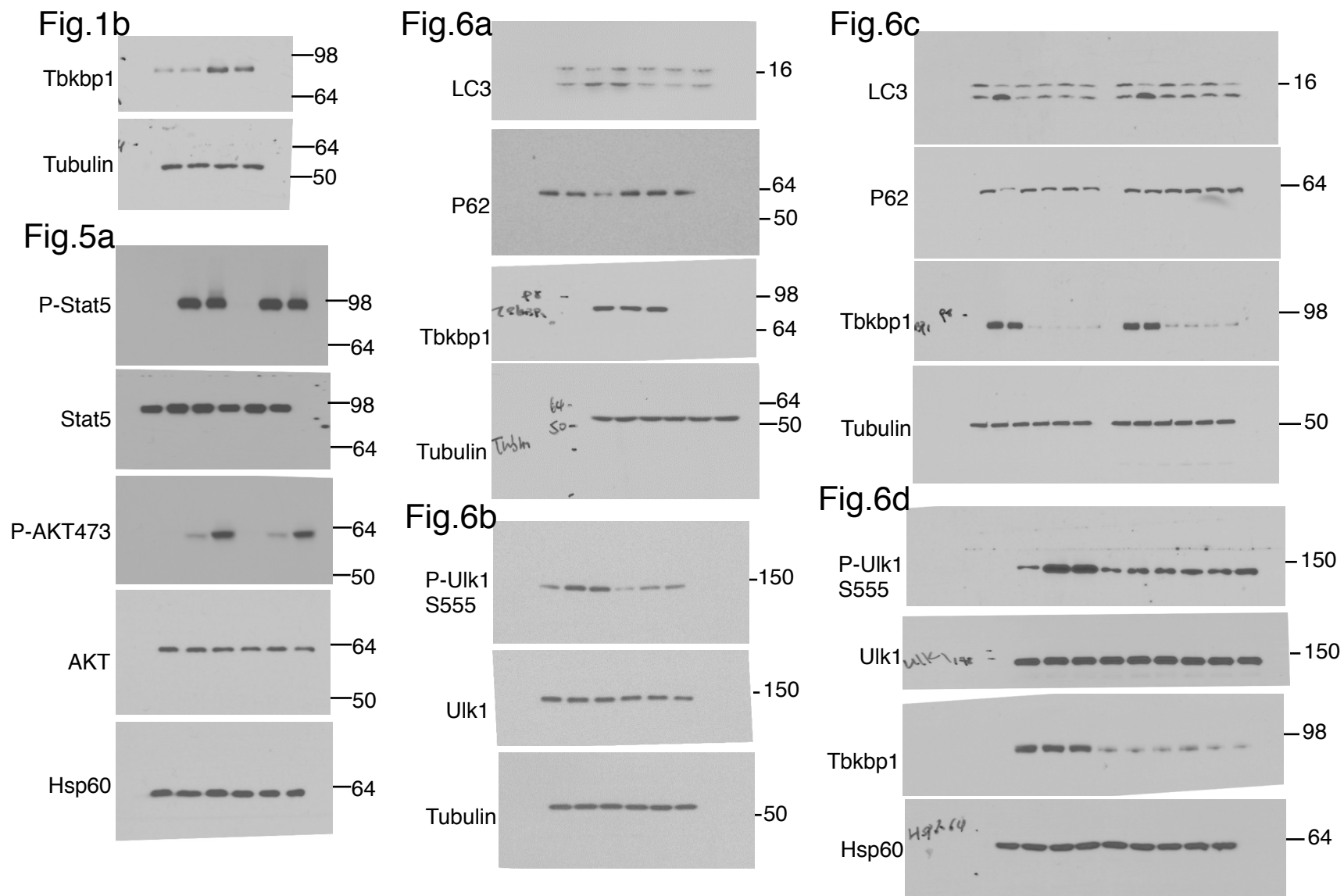
Supplementary Fig. 7. *Tbkbp1* is required for IL-15-mediated survival and autophagy of NK, but not memory CD8 $^{+}$ T, cells. **a,b**, Flow cytometric analysis of NK cell frequency and absolute numbers (**a**) and maturation (**b**) in the bone marrow (BM) and spleen of WT or *Tbkbp1*-KO (KO) mice, presented as representative plots (left) or summary graphs (right). Four progressive maturation stages of spleen NK cells are indicated in **b**. **c**, Flow cytometric analysis of splenic NK cell apoptosis in WT or *Tbkbp1*-KO (KO) that were either not treated (NT) or incubated with IL-15 for 48 h. **d**, IB analysis of the indicated phosphorylated (P-) and total proteins in IL-15-stimulated WT or *Tbkbp1*-KO (KO) NK cells. **e,f**, Memory CD8 $^{+}$ T cells were generated by stimulating WT or *Tbkbp1*-KO (KO) naïve CD8 $^{+}$ T cells with anti-CD3 plus anti-CD28 in the presence of IL-2 for 3 days and then cultured in the presence with IL-15 for 3 additional days. The cells were starved overnight and restimulated with IL-15 for 24 h (**e**) or for the indicated time periods (**f**) and then subjected to flow cytometric (**e**) and IB (**f**) assays. Student's t-test (**a,b,c,e**).



Supplementary Figure 8. Gating strategies used for cell sorting. **a**, Gating strategy to sort NKT cells from WT or *Tbkbp1* KO mice (Fig. 1a, 1b, 4g, 5a, 6a, 6b, 7a, and 7d). **b**, Gating strategy to sort NK cells from WT or *Tbkbp1* KO mice (Fig. 1a, 1b, and sFig 7d). **c**, Gating strategy to sort naïve CD8 cells from WT or *Tbkbp1* KO mice (sFig. 7e-f). **d,e**, Gating strategy to sort human CD4 cells, CD8 cells (d) or NKT cells (e) from *in vitro* PBMCs culture (sFig. 1a-c).



Supplementary Figure 9. Gating strategies used for flow cytometric analysis. a, Gating strategy for flow cytometric analysis of WT or Tbkbp1-KO (KO) NKT cells (Fig. 1c, 1d, 1f, 1g, 1h, 2a, 2c, 4a, 4c-f, 5b-e, 6f, sFig. 2b, 3d, 5a, 5c, 5e, 6c, 6h and 6i). **b,** Gating strategy to analyze splenic CD8 cells (Fig.3b and sFig.3b). **c,** Gating strategy to analyze thymic CD8 cells (Fig.3a, 3c, 3d and sFig.3a). **d,** Gating strategy for flow cytometric analysis of CD4 and CD8 T cells in tumors (Fig.3g, 3h and sFig 4b-e).



Supplementary Figure 10. Gel images for Figures 1b, 5a, and 6a-6d.

Fig.6h

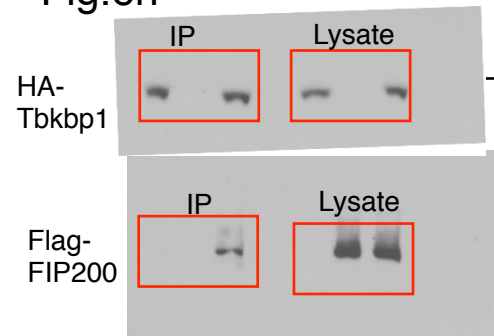


Fig.6j

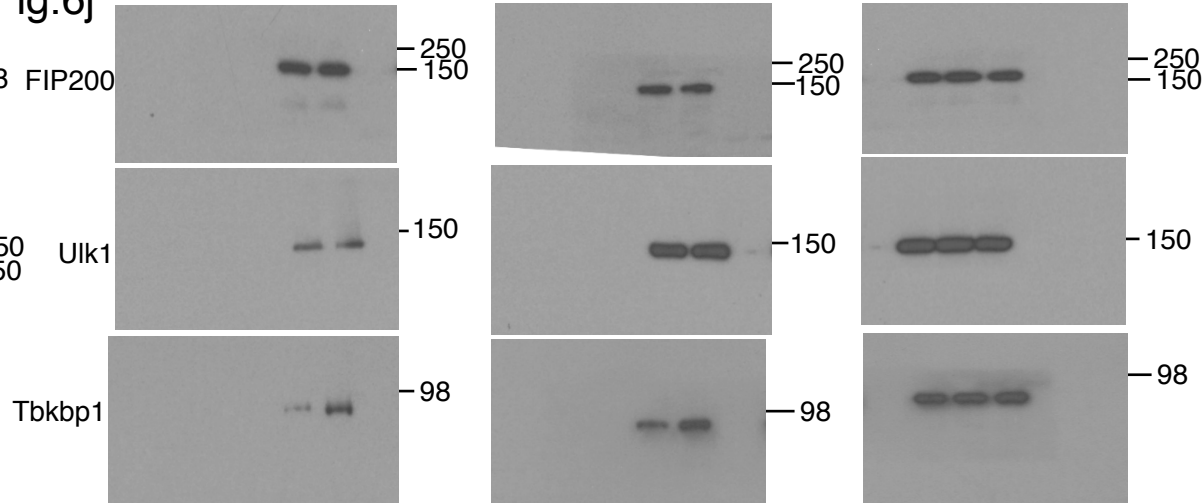


Fig.6i

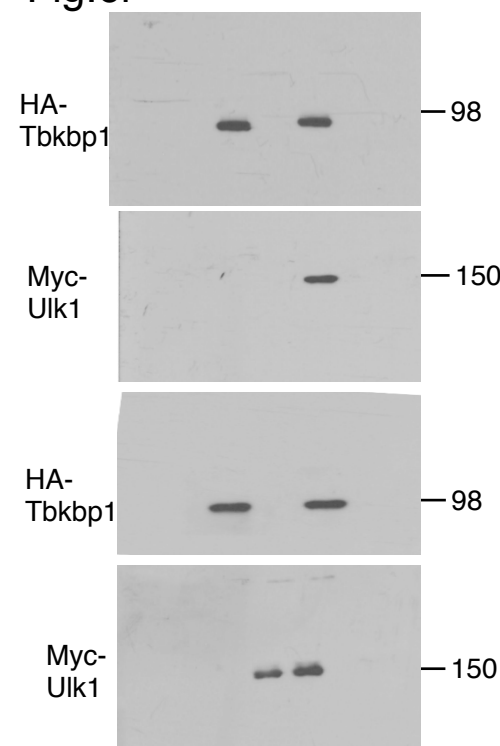


Fig.6l

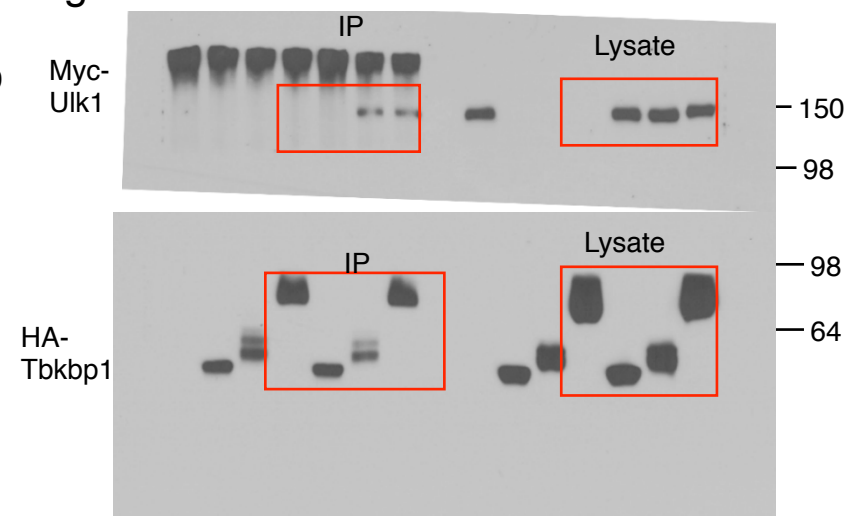
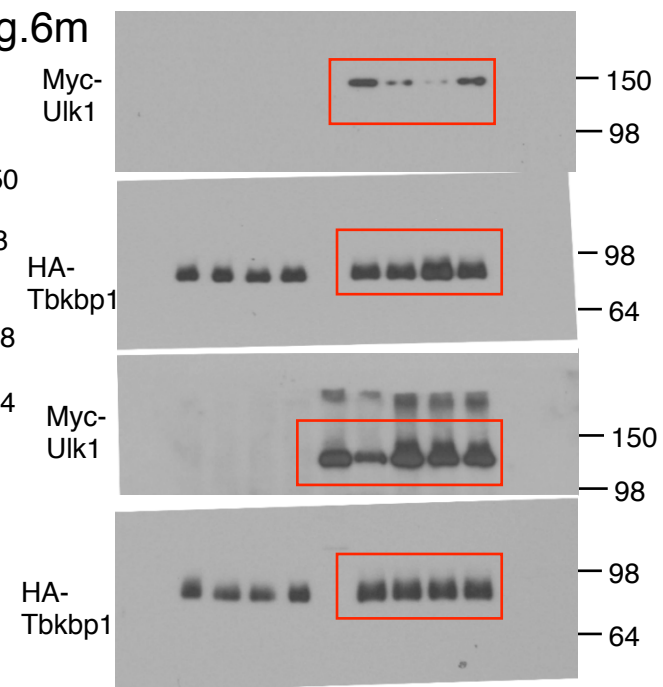


Fig.6m



Supplementary Figure 11. Gel images for Figures 6h-6m

Fig.6n

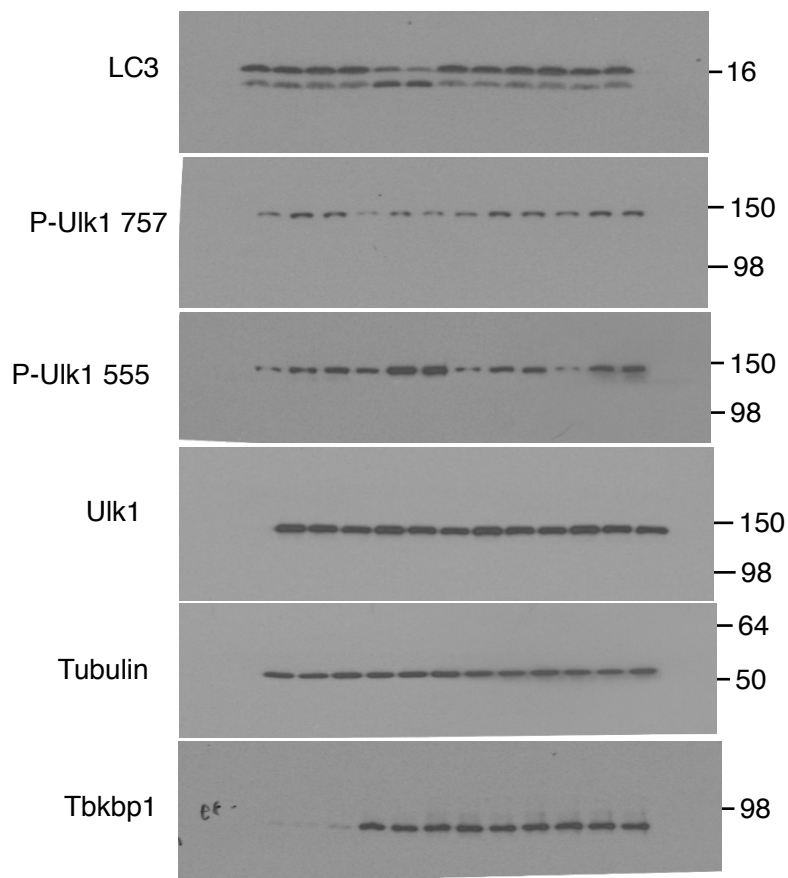


Fig.7a

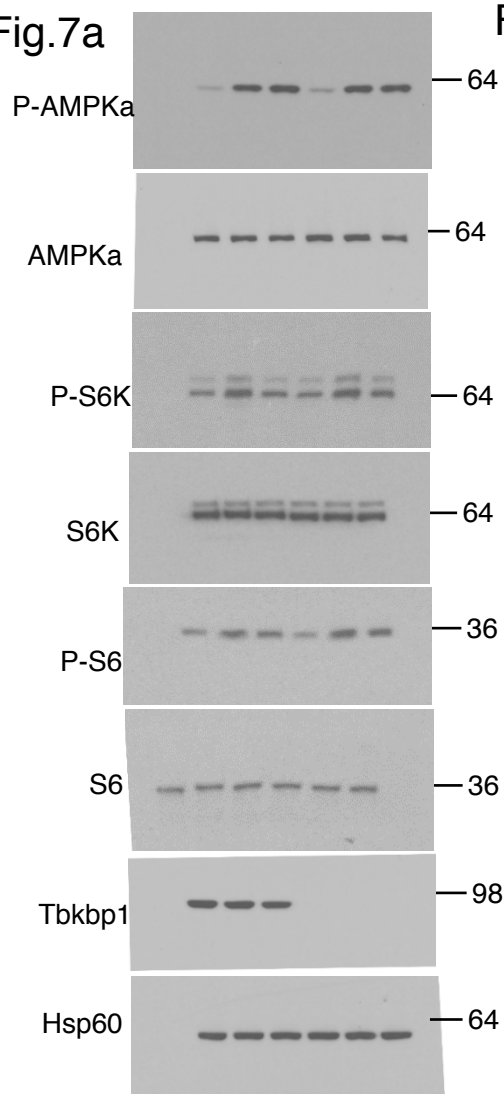
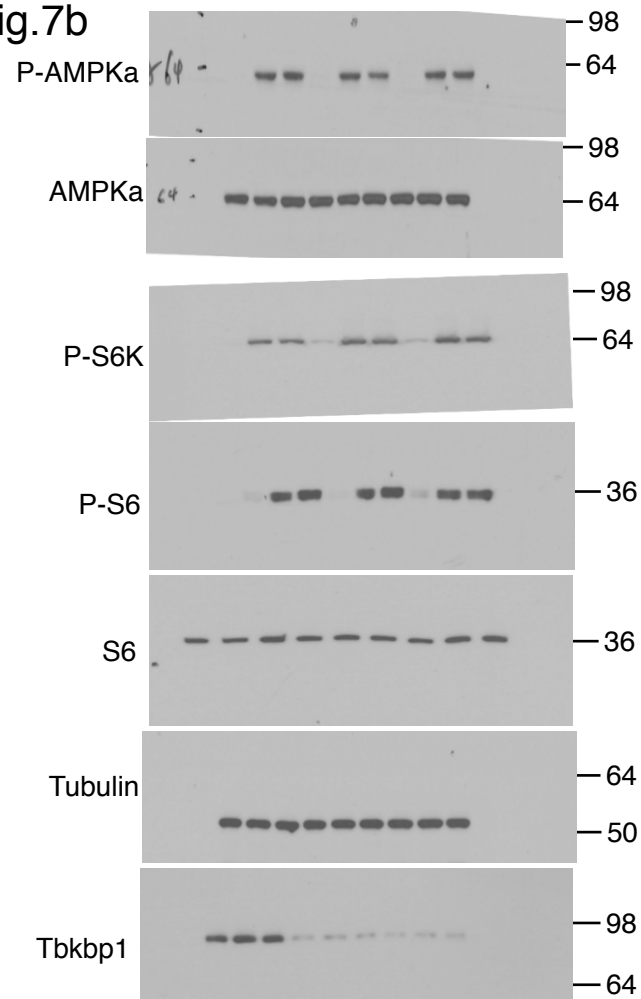
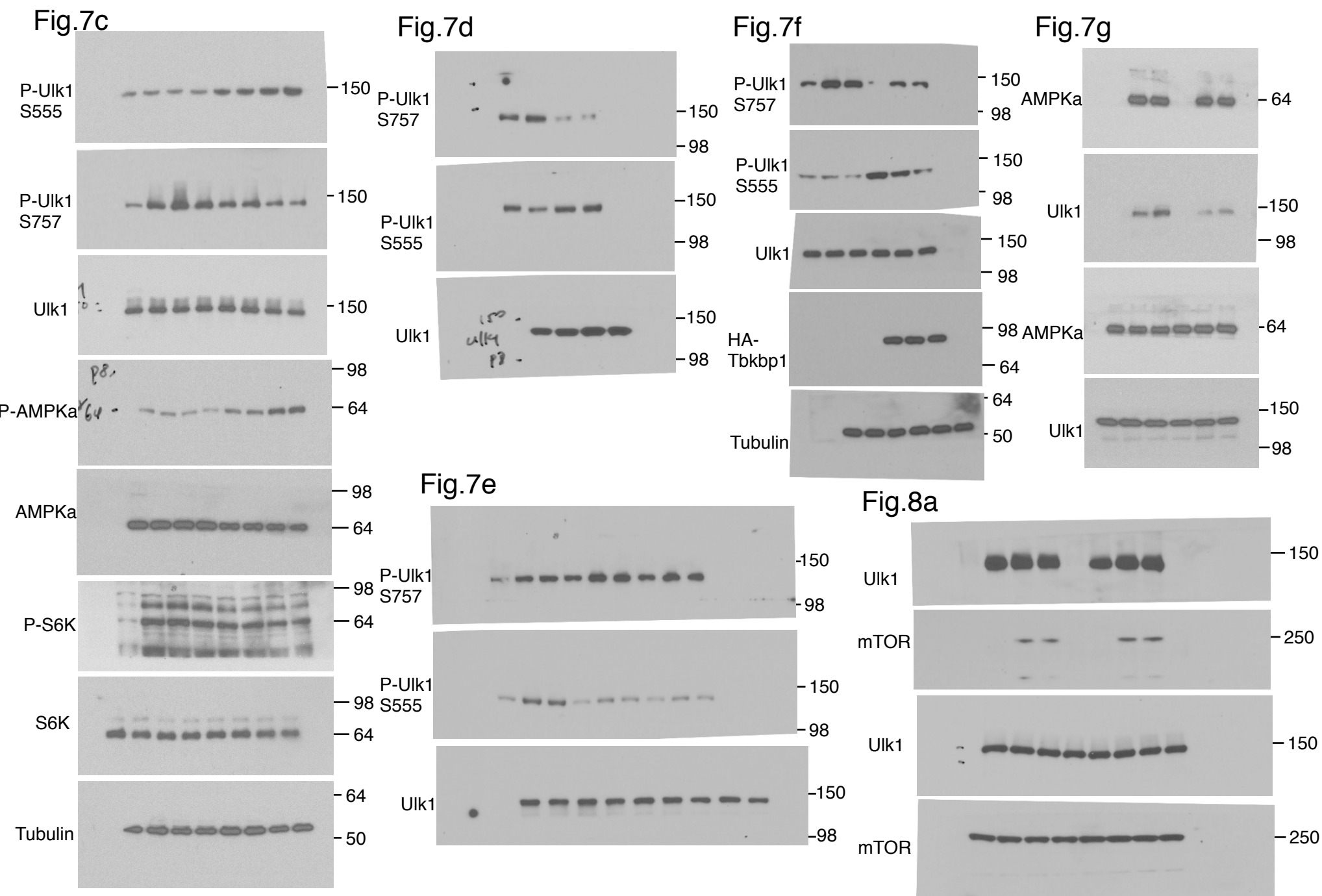


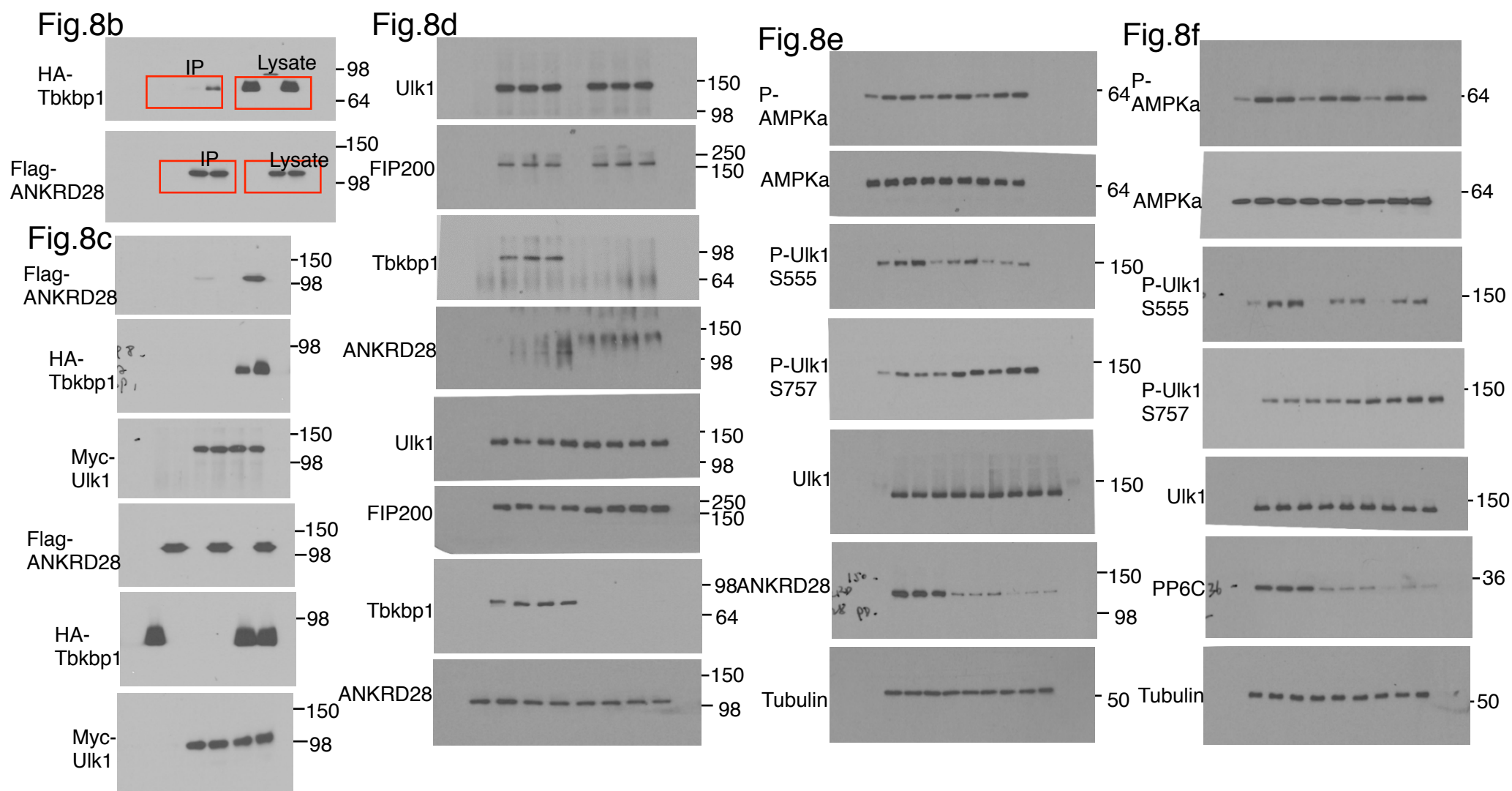
Fig.7b



Supplementary Figure 12. Gel images for Figures 6n, 7a, and 7b



Supplementary Figure 13. Gel images for Figures 7c-7g, 8a



Supplementary Figure 14. Gel images for Figures 8b-8f

Supplementary Table 1 Gene-specific primers used in qRT-PCR experiments

Gene	Forward primer	Reverse primer
mTbkbp1	GGCTTGGGTCAAGAGAGTTG	GTCCTGCAGCAAAGTCCTC
mBcl2	TCTGTGCACTGTGCATCTCTC	GACTTGGTGCATGGAACACTG
hTbkbp1	CTGATCAGTGACTTTGGAGAGG	CTCATGCTGGAAGTGGTTGA
mActin	CGTGAAAAGATGACCCAGATCA	CACAGCCTGGATGGCTACGT
hActin	CGAGGCCCAGAGCAAGAGAG	CGGTTGGCCTTAGGGTTCAG