Supplementary Information

Oncogenic potential of truncated RXRα during colitis-associated colorectal tumorigenesis by promoting IL-6-STAT3 signaling

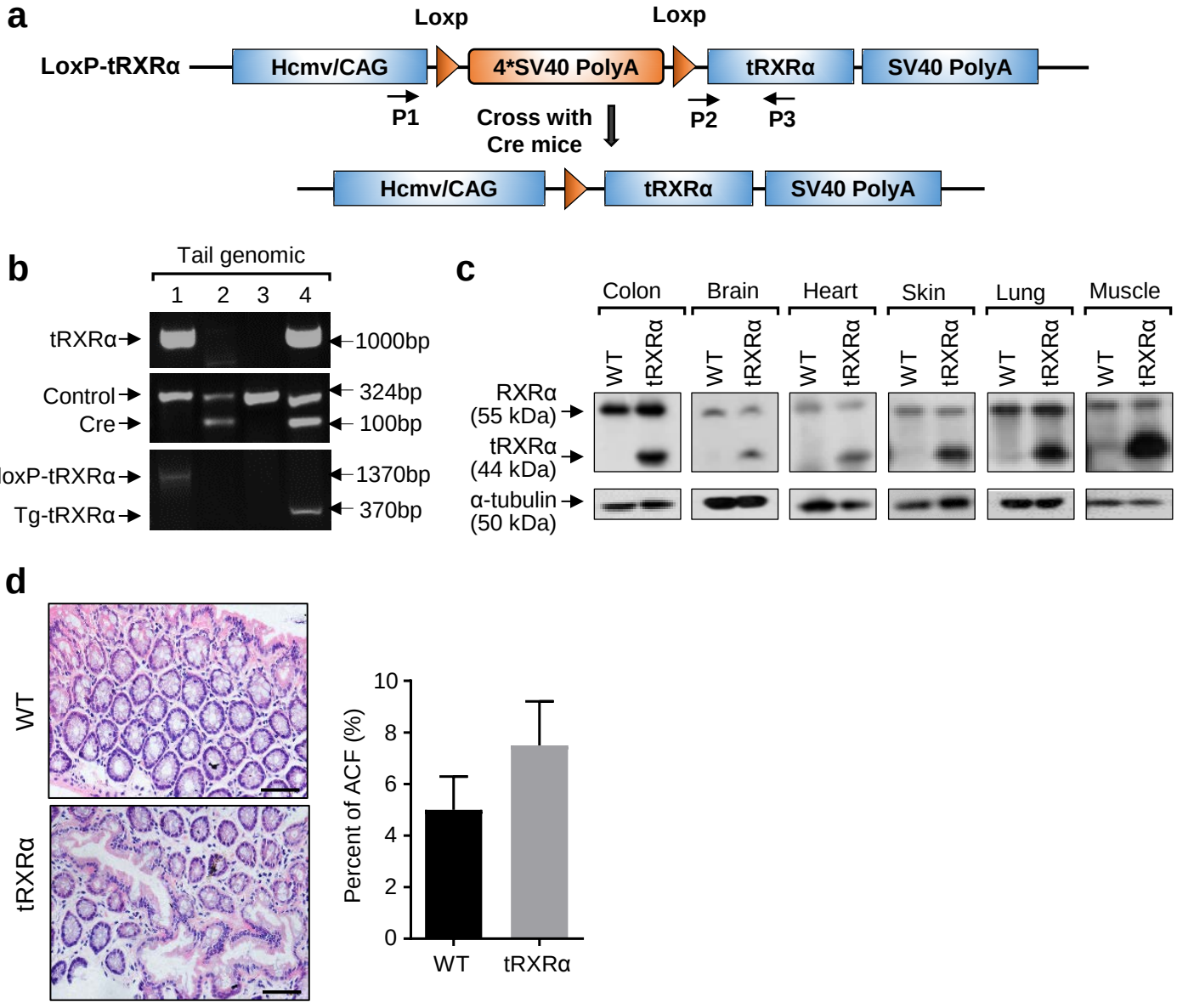
Ye et al.

Supplementary Table 1

Supplementary Table 1. Primers used for quantitative RT-PCR.

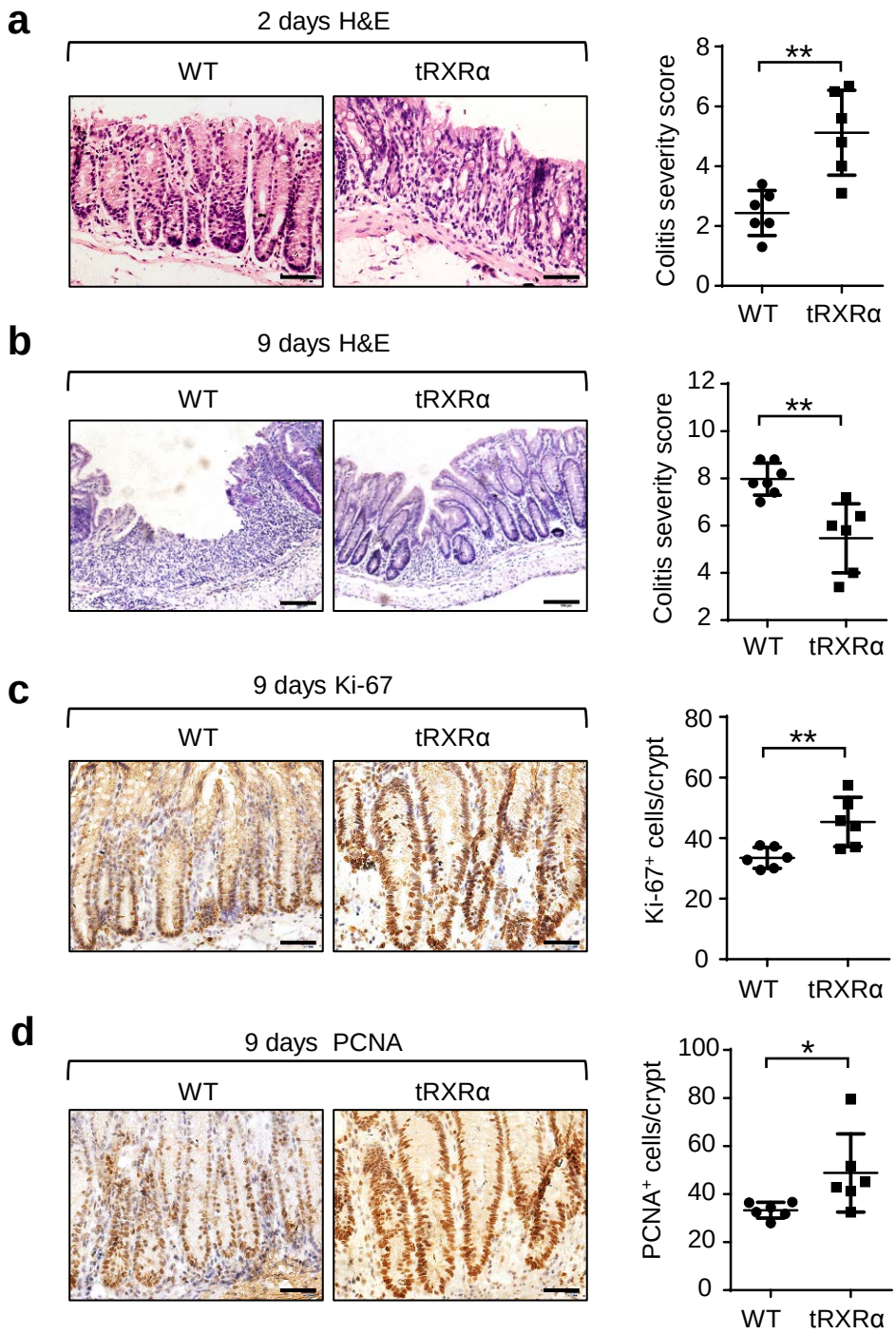
Gene	Forward primers (5’-3’)	Reverse primers (5’-3’)
GAPDH	TTGATGGCAACAATCTCCAC	CGTCCCGTAGACAAAATGGT
TNFα	AGGGTCTGGGCCATAGAACT	CCACCACGCTCTTCTGTCTAC
IL-6	CTCTGCAAGAGACTTCCATCCAGT	GAAGTAGGGAAGGCCGTGG
IL-11	TGTTCTCCTAACCCGATCCCT	CAGGAAGCTGCAAAGATCCCA
Cyclin D2	GCGTGCAGAAGGACATCCA	CACTTTTGTTCCTCACAGACCTCTAG
RegIIIγ	TGCTGCTCTCCTGCCTGATG	ATAGGAGCCATAGGCACGGG
Tff3	TTGCTGGGTCCTCTGGGATAG	TACACTGCTCCGATGTGACAG

Supplementary Figure 1



Supplementary Figure 1. Generation and characterization of *Tg-tRXRα* mice.
(**a**) Schematic representation of the production of *Tg-tRXR* mice. Mouse tRXRα cDNA cloned into the pCMV/CAG-loxp/4*SV40/loxp plasmid to generate a transgene vector. Transgenic mice on a C57BL/6 background were generated by pronuclear microinjection, which were crossed with Cre mice to generate Tg-*tRXRα* transgenic mice. P1,P2,and P3 primers were designed to detect loxP-tRXRα and Tg-tRXRα.
(**b**) Tail genomic analysis of Tg-*tRXRα* mouse by PCR. 1, loxP-tRXRα; 2, CMV-cre; 3, wild-type; 4, Tg-tRXRα. The presence of tRXRα sequences in loxP-tRXRα and Tg-tRXRα mice was illustrated by using P2 and P3 primers. By using P1 and P3 primers, the bands of loxP-tRXRα (1370 bp) and Tg-tRXRα (370bp) were detected, demonstrating the deletion of loxp-SV40-loxp sequences from *Tg-tRXRα* mouse.
(**c**) Expression of tRXRα in different tissues from loxP-*tRXRα* (WT) and *Tg-tRXRα* mice was determined by immunoblotting.
(**d**) Representative H&E images of colon sections from *loxP-tRXRα* (WT) and *Tg-tRXRα* mice (21-month old) and examined for microscopic aberrant crypt foci (ACF) after H&E. Data are mean ± SEM (n=6), Scale bar, 50 μm.

Supplementary Figure 2

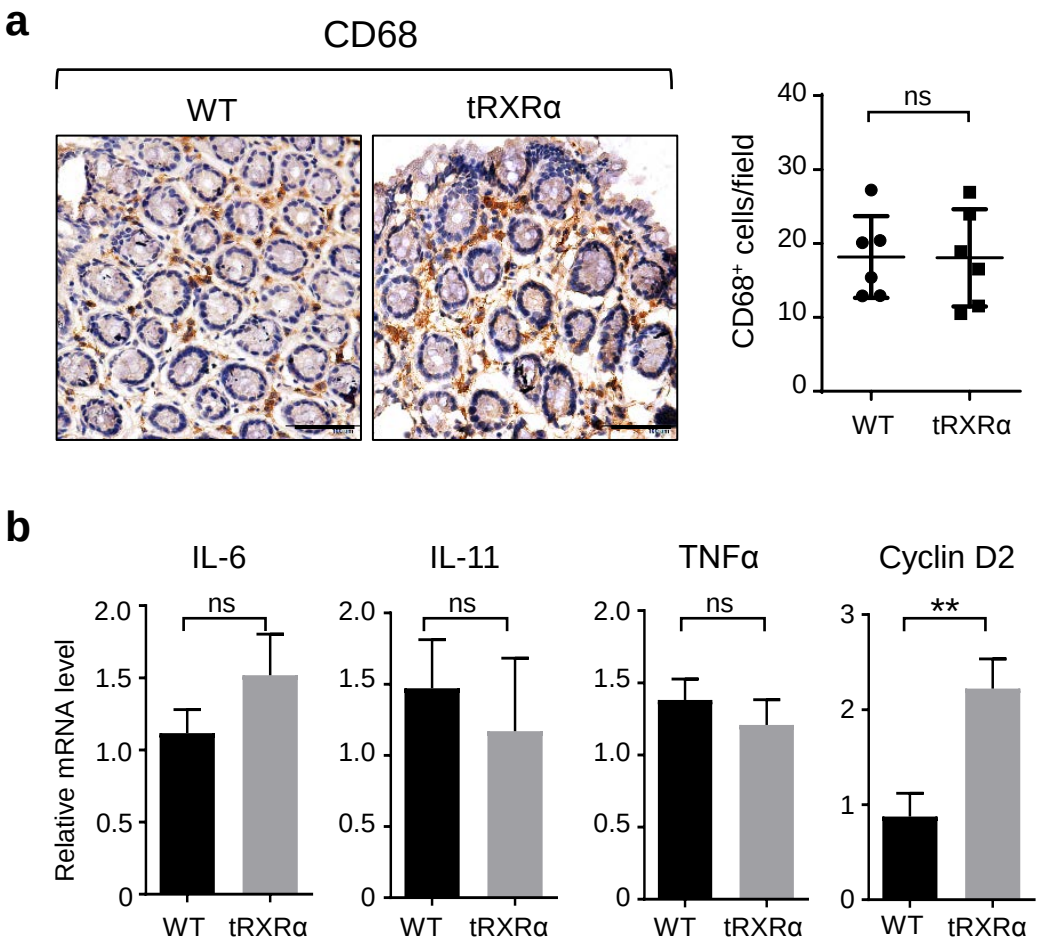


Supplementary Figure 2. tRXRα promotes inflammation and intestinal cell proliferation.

(**a, b**) Representative H&E images of colon sections from mice treated with 3%DSS for 2 days (**a**) or with 3%DSS for 5 days followed by 4 days recovery (**b**). Crypt damage with histopathology was scored. Data are mean ± SD (n=6), ** $p < 0.01$ by t test. Scale bar, 100 μm.

(**c, d**) Representative micrographs of colonic sections from mice administrated with 3%DSS for 5 days followed by 4 days recovery were stained for Ki-67 (**c**) or PCNA (**d**). (scale bar, 100μm) and analyzed The numbers of Ki-67-positive or PCNA-positive cells per crypt were analyzed by counting 18-20 full-length crypts per mouse colon. Data are mean ± SD (n=6). *$p < 0.05$; **$p < 0.01$ by t test. Scale bar, 100μm.

Supplementary Figure 3

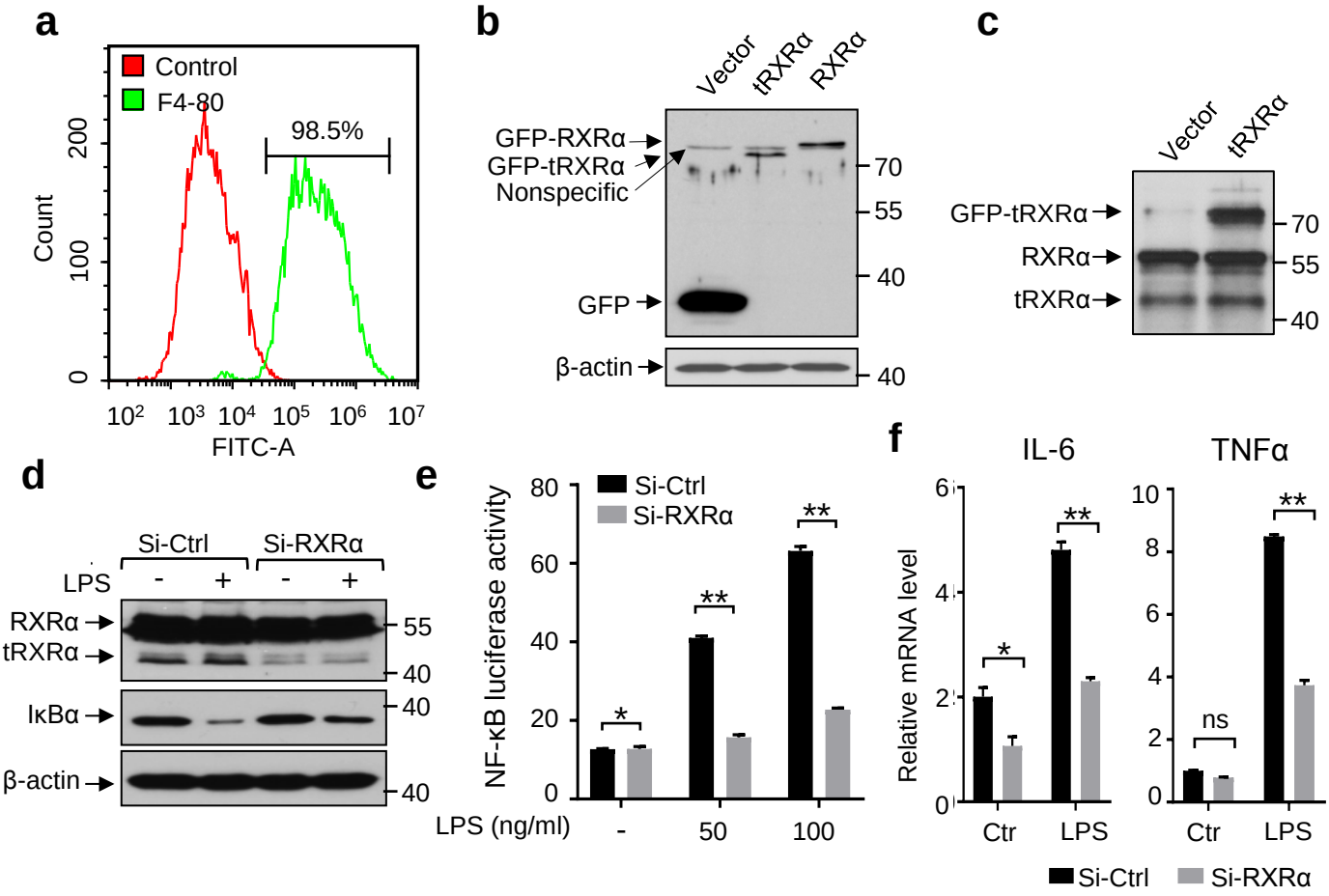


Supplementary Figure 3. tRXRα does not promote inflammation in AOM-induce colorectal carcinogenesis.

(**a**) Representative micrographs of colonic sections from mice treated with the AOM protocol for 6 months were stained for CD68 and analyzed the number of CD68-positive cells per field. Each dot represents a mouse. 10 fields were counted per mice. Data are mean ± SD (n=6); ns, no significant. Scale bar, 100 μm.

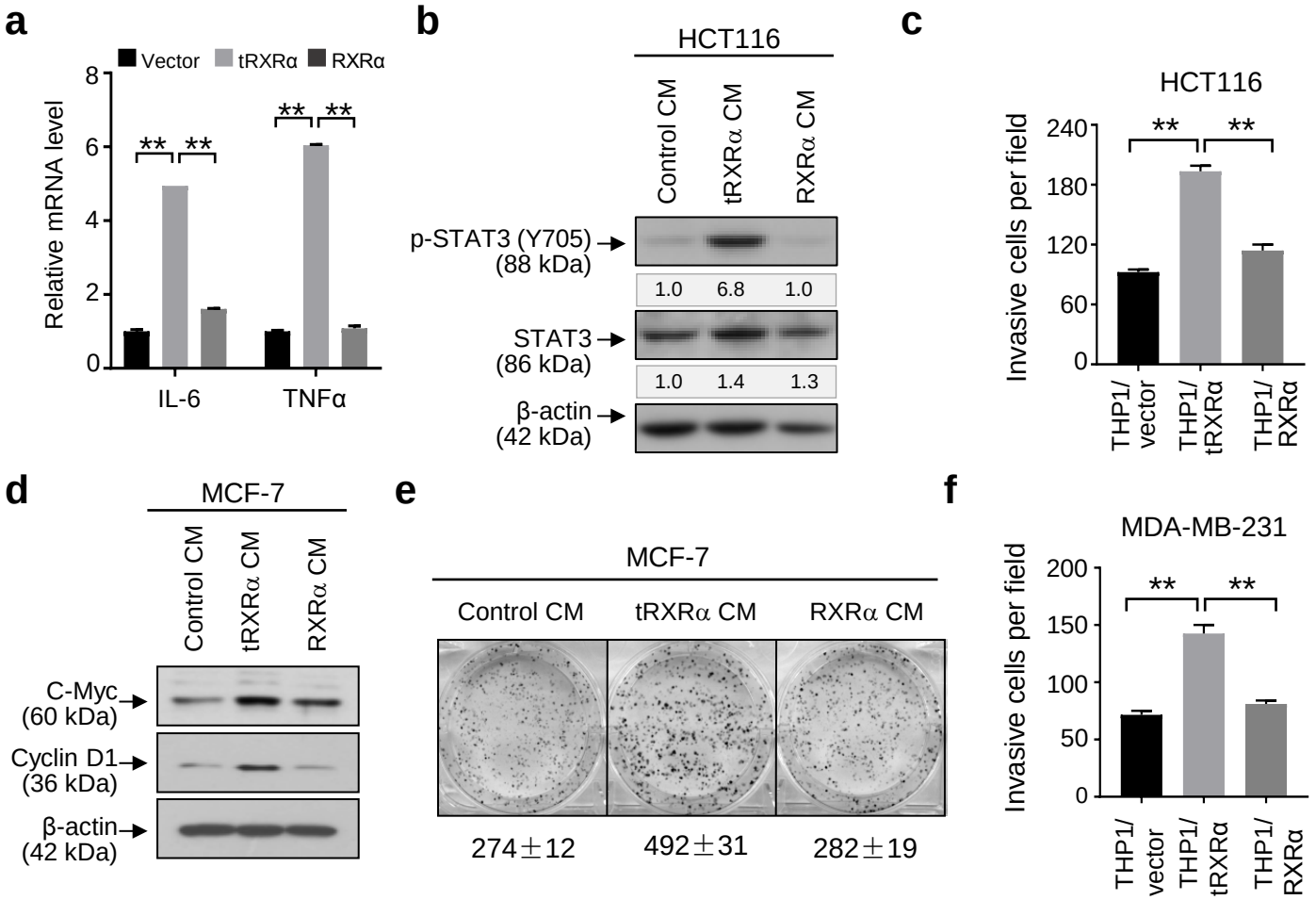
(**b**) Relative mRNA expression levels of the indicated genes in colon tissue from mice treated with the AOM protocol for 6 months were determined by qRT-PCR. Data are mean ± SEM (n = 5), $^{**}p < 0.01$ by t test; ns, no significant.

Supplementary Figure 4



Supplementary Figure 4. tRXRα activation of the IKK-NF-κB pathway in macrophages.
(**a**) Flow cytometric analysis of the purity of BMDMs from bone marrow, which were differentiated and treated with or without macrophages marker F4/80 (green).
(**b,c**) Stable expression of tRXRα and RXRα in RAW264.7 cells. The GFP-tRXRα, GFP-RXRα or GFP Vector plasmid were transfected into RAW264.7 cells, and stable clones were selected with G418 (600 μg/ml) and analyzed by immunoblotting using anti-GFP antibody (**b**) or ΔN197 anti-RXRα antibody in order to compare the expression level of transfected and endogenous tRXRα (**c**).
(**d**) Lysates from THP-1 cells transfected with control or RXRα siRNA were treated with LPS (50 ng/ml) for 30 min and analyzed by immunoblotting.
(**e**) THP-1 cells transfected with control or RXRα siRNA were treated with LPS and analyzed for NF-κB transactivation by reporter assay.
(**f**) Expression of IL-6 and TNFα mRNAs in THP-1 cells transfected with control or RXRα siRNA and treated with or without LPS was analyzed by RT-PCR.
Data are mean ± SEM. *$p < 0.05$, **$p < 0.01$ by two-way ANOVA.

Supplementary Figure 5



Supplementary Figure 5. tRXRα activation of the IKK–NF-κB pathway in macrophages.

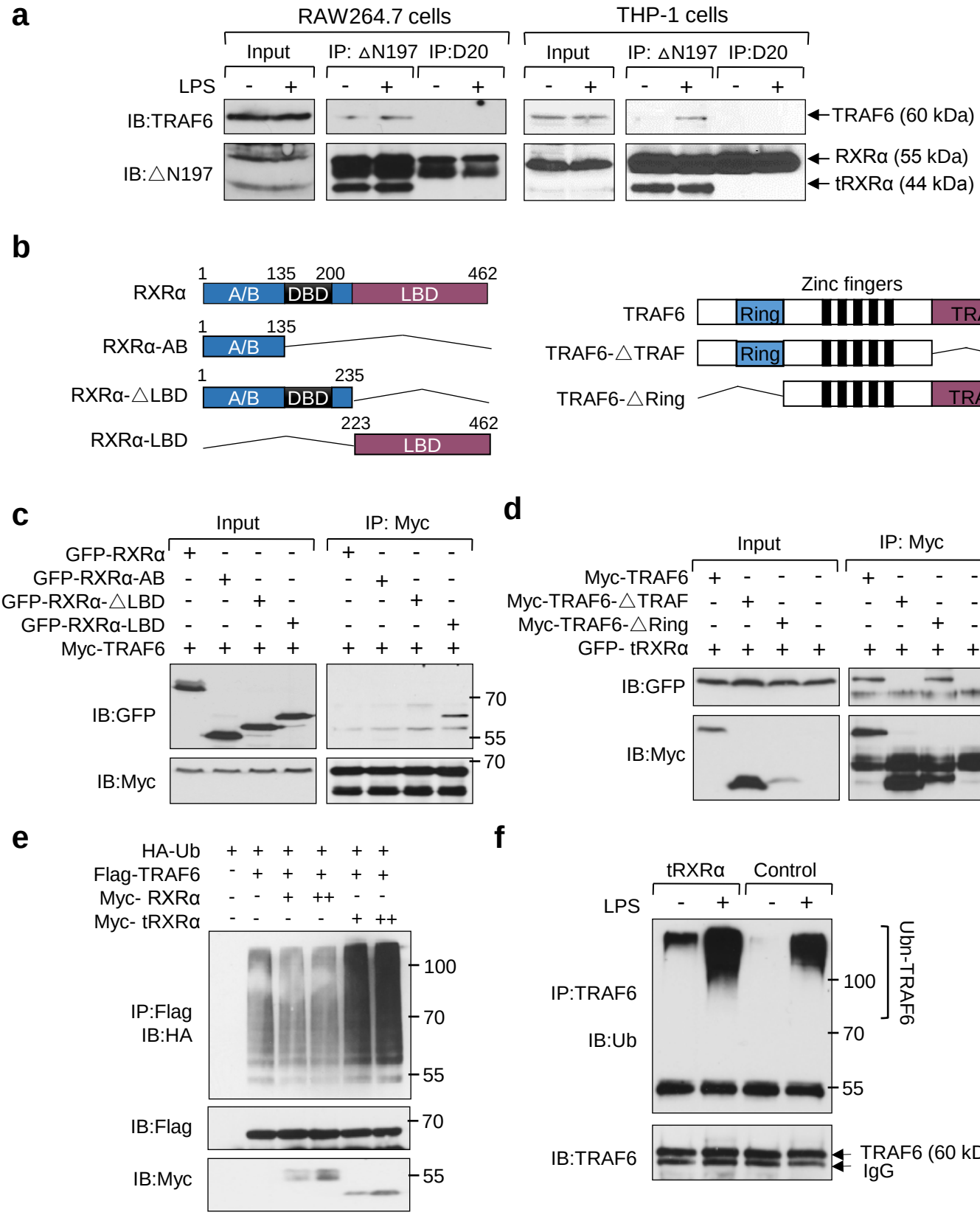
(**a**) Relative mRNA expression of IL-6 and TNFα in THP-1 cells transfected with tRXRα or RXRα were determined by qRT-PCR.

(**b,c**) Effect of CM from THP-1 cells transfected with tRXRα or RXRα on STAT3 activation on HCT116 colon cancer cells and their invasion. The expression of pSTAT3 and STAT3 was determined by immunoblotting (**a**) and the invasive ability of HCT116 cells upon co-culture was determined by invasion assay (**b**).

(**d,e**) Effect of CM from THP-1 cells transfected with tRXRα or RXRα on STAT3 activation of breast cancer cells and their growth. MCF-7 cells cultured with CMs for 24 h were analyzed for gene expression by immunoblotting (**c**). Alternatively, they were cultured with CMs for 2 weeks and analyzed for their colony formation (**d**). The number of colonies was counted per dish.

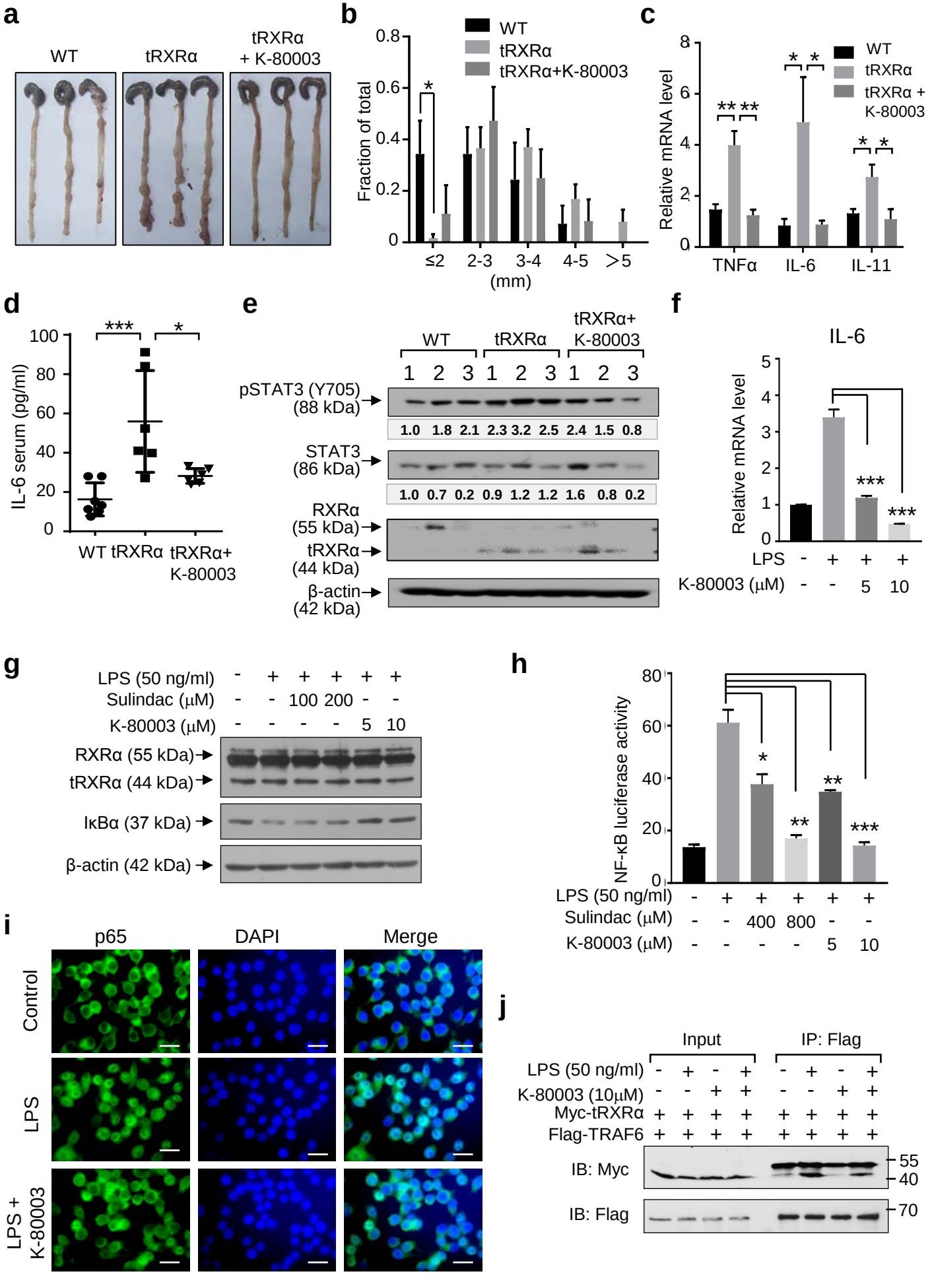
(**f**) THP-1 cells expressing tRXRα promotes the invasion of MDA-MB-231 breast cancer cells. MDA-MB-231 breast cancer cells were co-cultured with THP-1 cells expressing tRXRα or RXRα, and their invasive ability was determined by in vitro invasion assay. Data are mean ± SEM. ** $p < 0.01$ by one-way ANOVA.

Supplementary Figure 6



Supplementary Figure 6. Characterization of tRXRα interaction with TRAF6.
(**a**) RAW264.7 cells and THP-1 cells treated with LPS (50 ng/ml) for 20 min were analyzed for tRXRα interaction with TRAF6 by co-immunoprecipitation assays using D20 and ΔN197 anti-RXRα antibodies. Immunoprecipitates were analyzed by immunoblotting (IB).
(**b**) Schematic representations of RXRα and TRAF6 and their mutants.
(**c**) LBD of RXRα is important for its interaction with TRAF6. The mutants of RXRα vectors were transfected into HEK293T cells and analyzed by immunoprecipitation using anti-Myc antibody.
(**d**) TRAF domain of TRAF6 is required for its interaction with tRXRα. The mutants of TRAF6 vectors were transfected into HEK293T cells and analyzed by immunoprecipitation using anti-Myc antibody.
(**e**) tRXRα but not RXRα promoted TRAF6 ubiquitination. HEK293T transfected with HA-Ub, Flag-TRAF6 together with Myc-RXRα or Myc-tRXR plasmids were immunoprecipitated with anti-Flag antibody. Immunoprecipitates were then analyzed for TRAF6 ubiquitination by immunoblotting.
(**f**) RAW264.7 cells stably expressing GFP or GFP-tRXRα were treated with or without LPS (50 ng/ml) for 30 min were immunoprecipitated with anti-TRAF6 antibody and analyzed for TRAF6 ubiquitination by immunoblotting.

Supplementary Figure 7



Supplementary Figure 7. Effect of K-80003 on inhibiting tRXRα-dependent development of CAC.

(**a**) Representative images of colon tumors from mice treated with AOM/DSS in the presence or absence of K-80003.

(**b**) Tumor size distribution. Data are mean ± SEM (n = 7/5), by two-way ANOVA.

(**c**) Relative mRNA expression levels of TNFα, IL-6 and IL-11 were determined by qRT-PCR. Data are mean ± SEM (n≥4), by one-way ANOVA.

(**d**) Serum levels of IL6 from mice treated with 3%DSS and/or K-80003 (20 mg/kg) were measured by ELISA. Each dot represents a mouse. . Data are mean ± SD (n = 6), by one-way ANOVA.

(**e**) Colon lysates from mice treated with AOM/DSS and vehicle control or K-80003 were analyzed by immunoblotting. The bands were quantified with image J and normalized to β-actin.

(**f**) Relative mRNA expression of IL-6 in RAW264.7 cells upon treatment with LPS and/or the indicated concentrations of K-80003 for 3 h was analyzed by RT-PCR. Data are mean ± SEM, by one-way ANOVA.

(**g**) RAW264.7 cells treated with the indicated concentrations of K-80003 or Sulindac for 1 hr before exposed to LPS (50 ng/ml) for 30 min were analyzed by immunoblotting.

(**h**) RAW264.7 cells transfected with pNF-κB reporter plasmid were treated with LPS, Sulindac, or K-80003, and analyzed for NF-κB transactivation by reporter assay. Data are mean ± SEM, by one-way ANOVA.

(**i**) RAW264.7 cells pretreated with K-80003 (10 μM) for 1 h before exposed to LPS (50 ng/ml) for 30 min were immunostained with anti-p65 antibody. Scale bar, 10 μm.

(**j**) RAW264.7 cells transfected with Flag-TRAF6 and Myc-tRXRα were treated with K-80003 (10 μM) for 1 h before exposed to LPS (50 ng/ml) for 30 min, and their interaction was analyzed by co-immunoprecipitation assay.

*P<0.05, ** P<0.01, ***p<0.001.

Fig. 2a

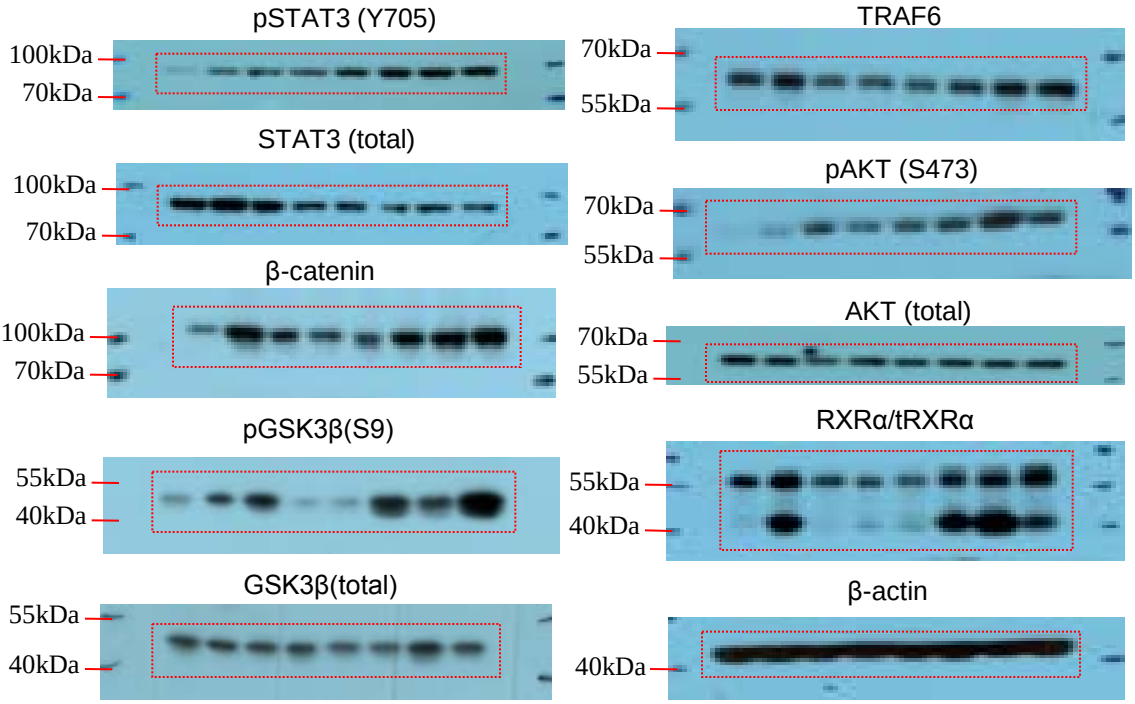


Fig. 2b

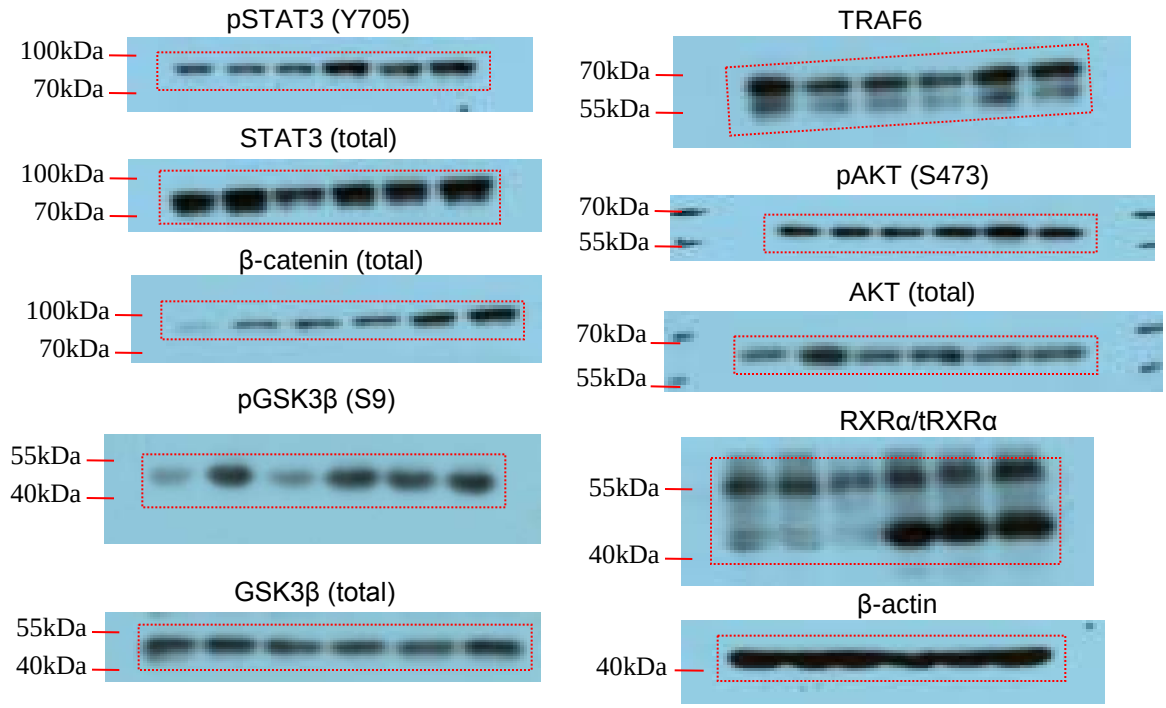


Fig. 2f

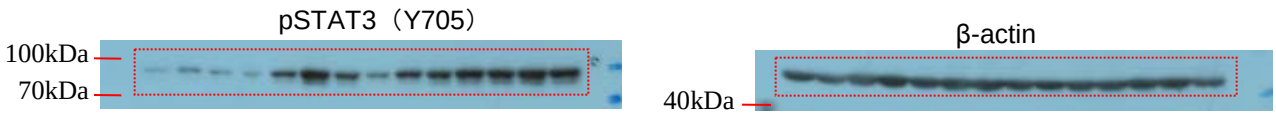
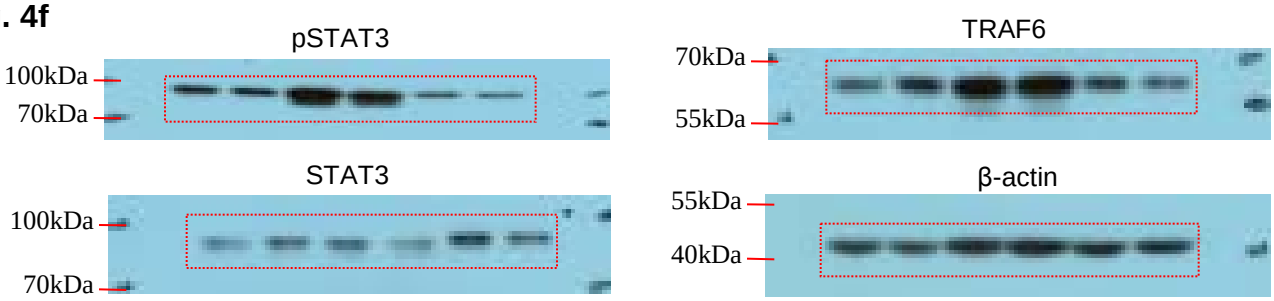


Fig. 4f



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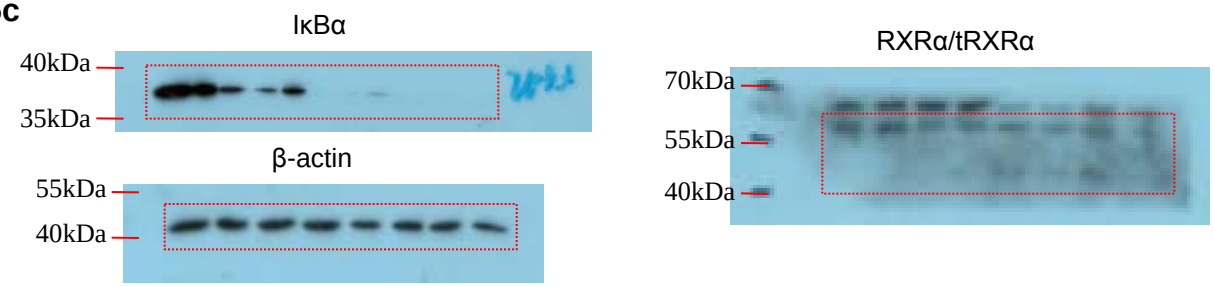

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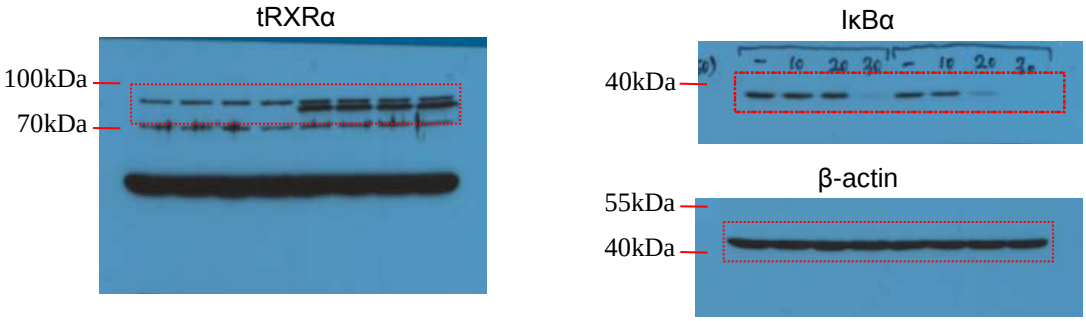

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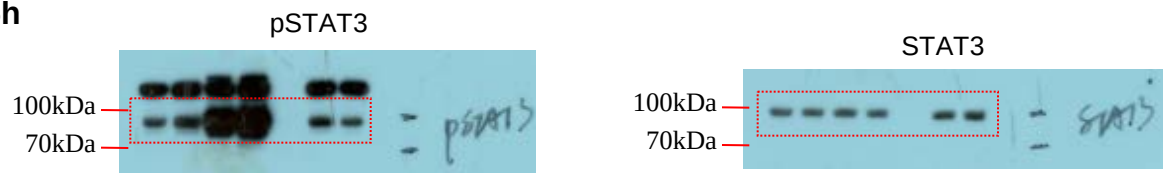
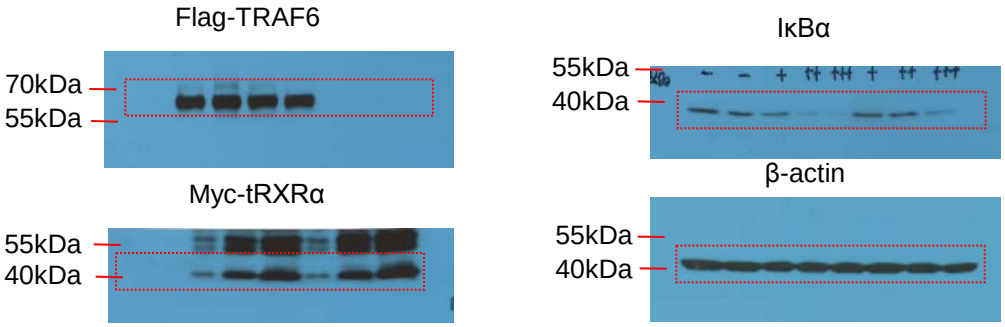


Fig. 6a



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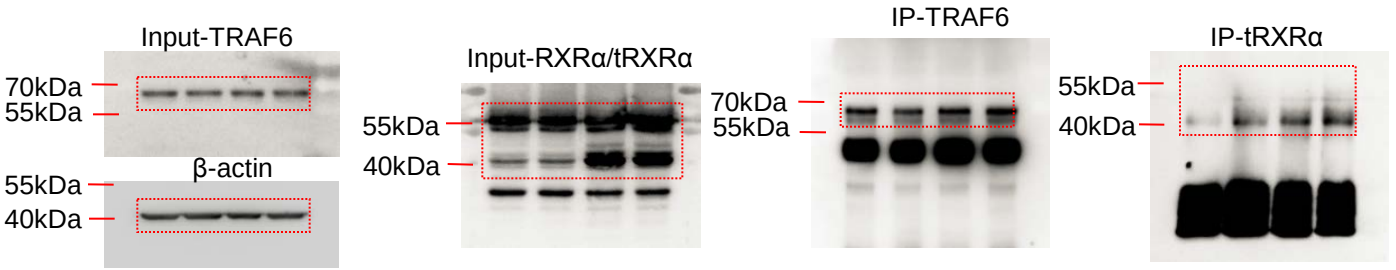

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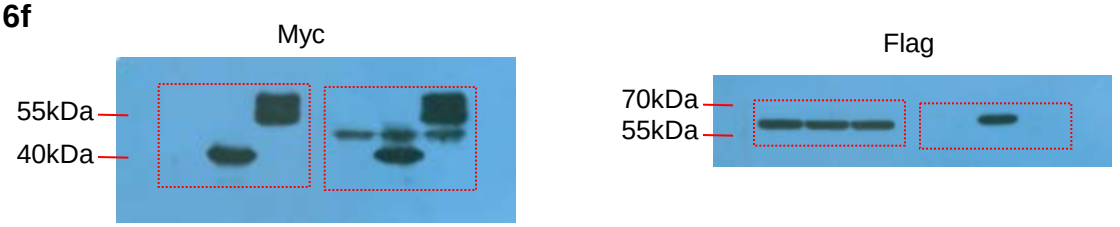

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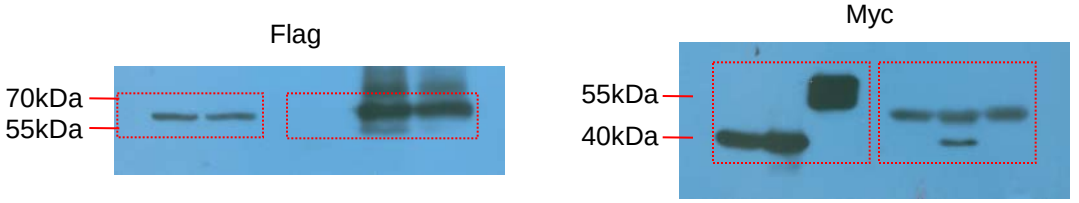

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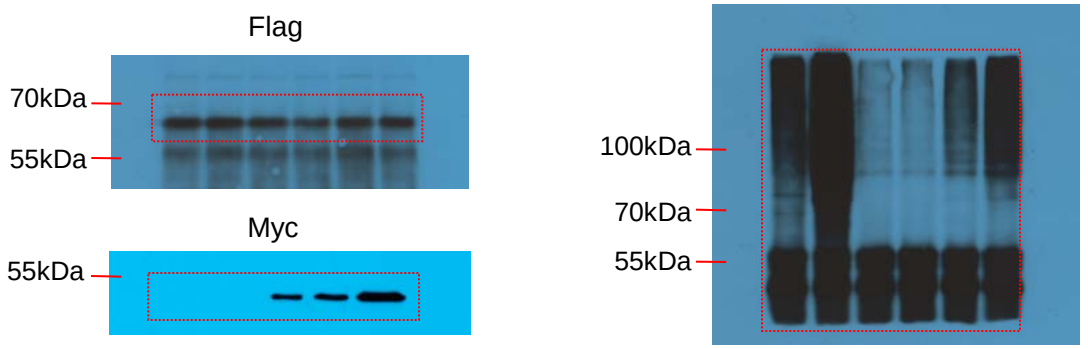


Fig. 6i

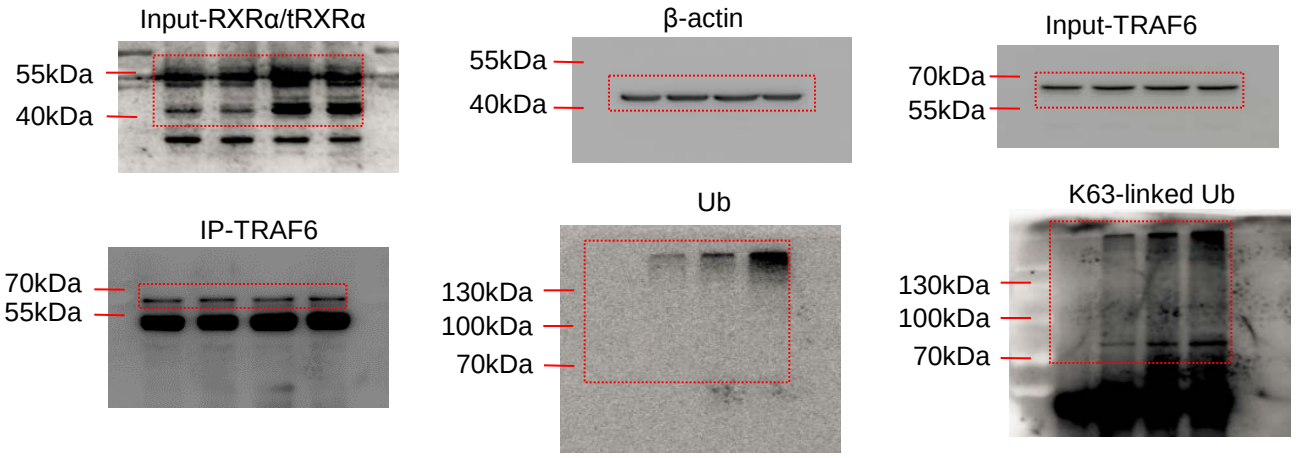
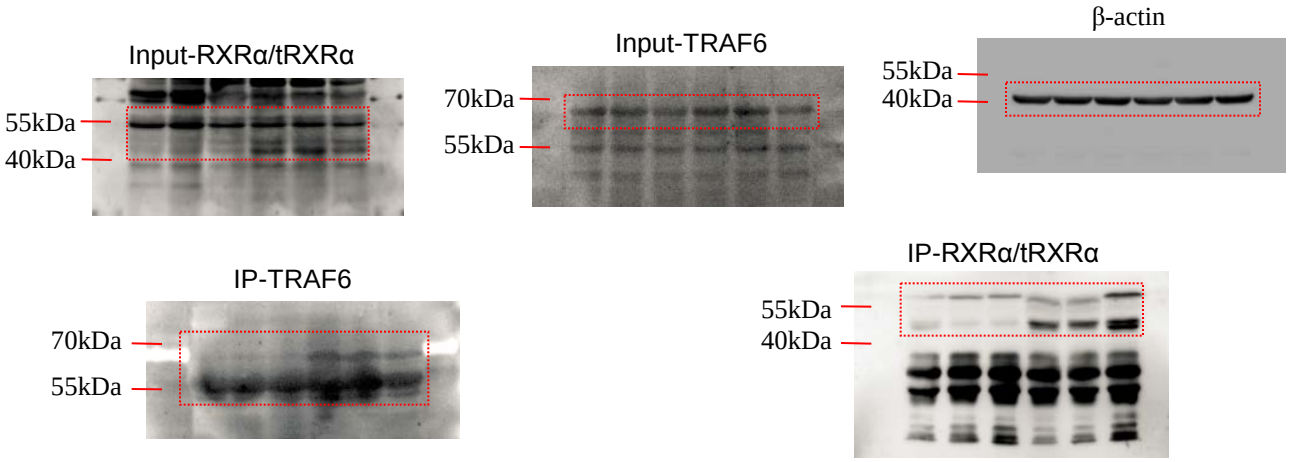


Fig. 7a



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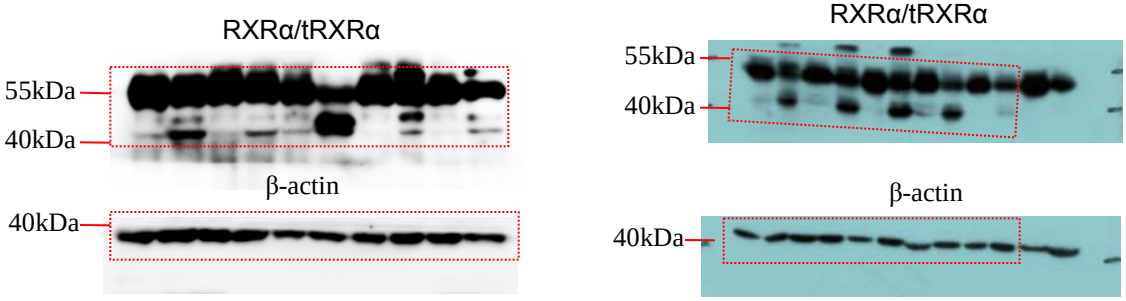
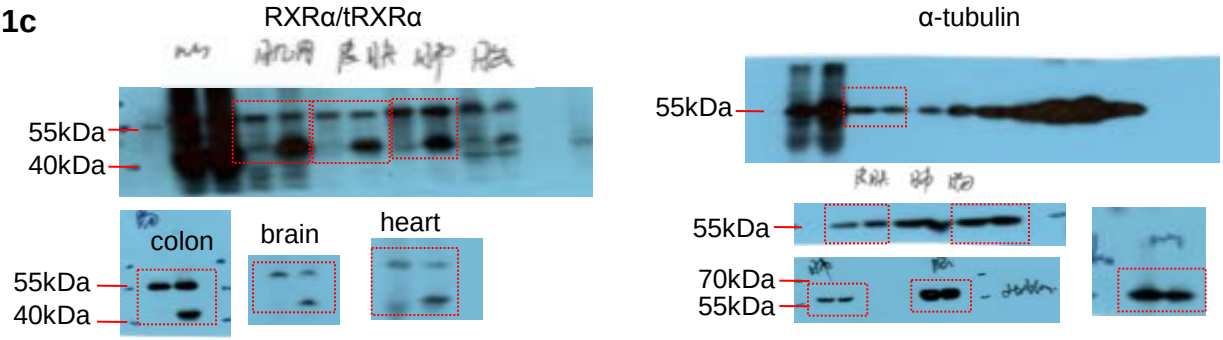


Fig. 7g

Fig. S1c



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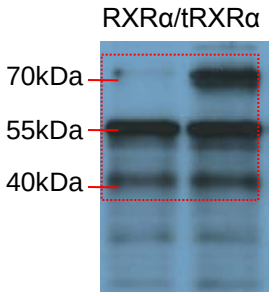

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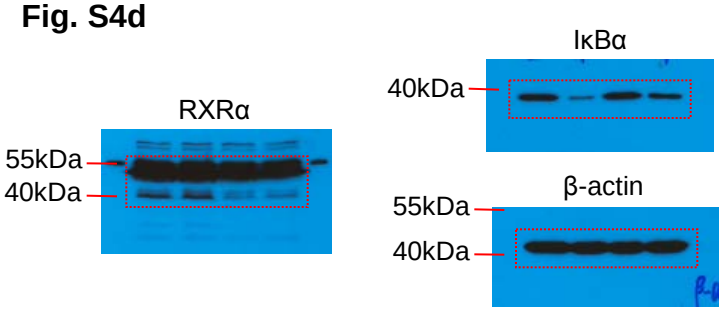

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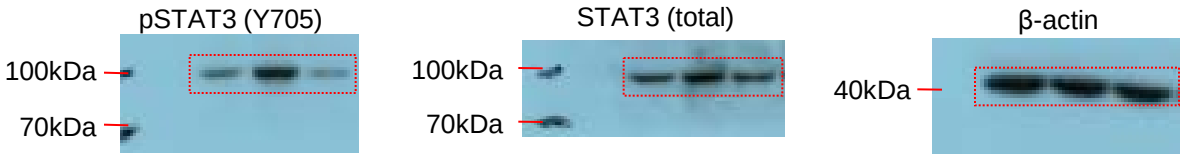

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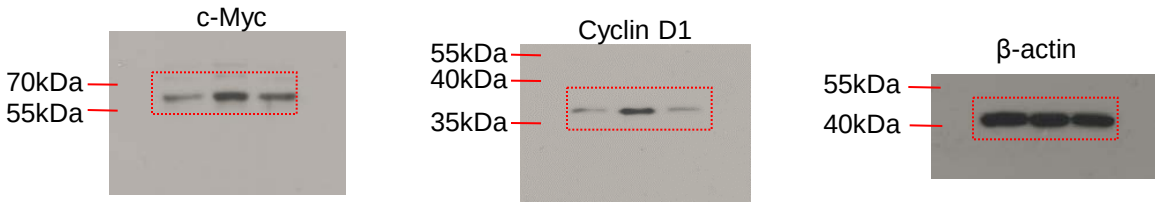

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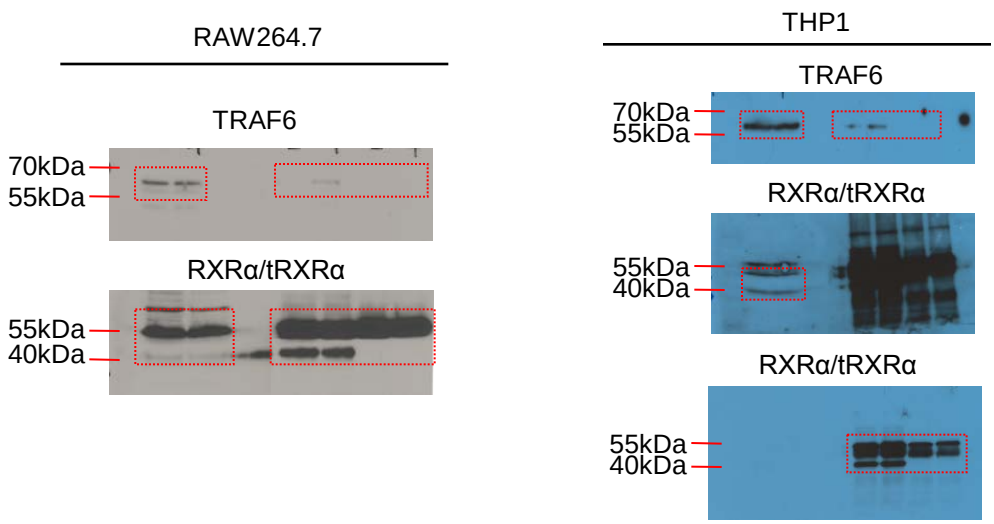

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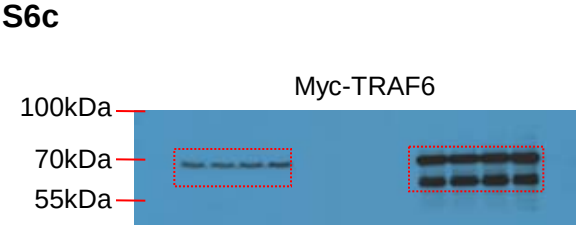

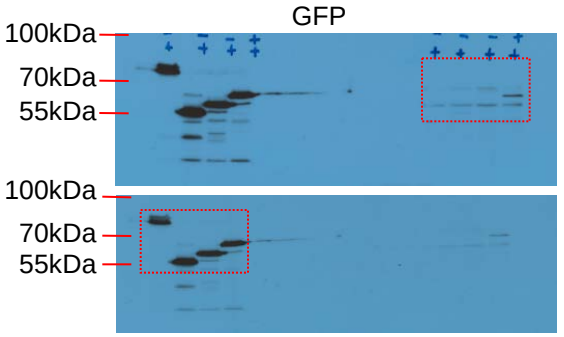

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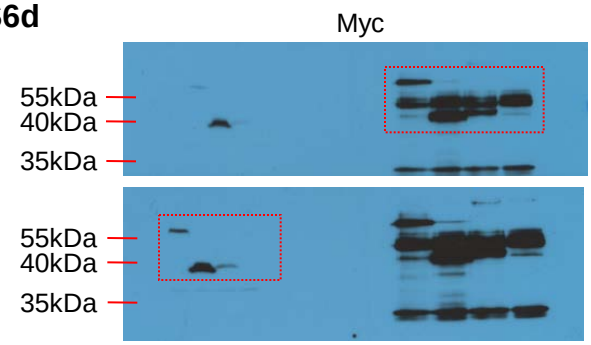

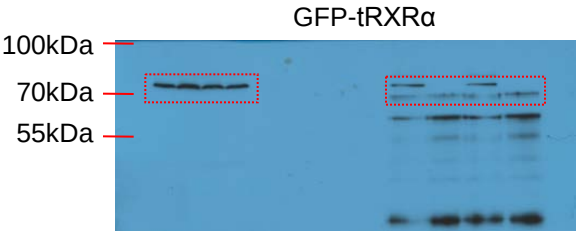

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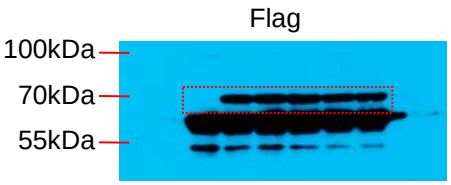

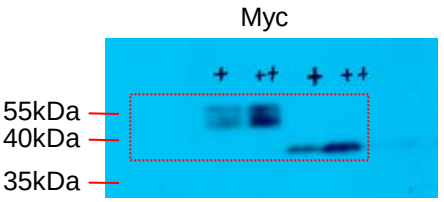

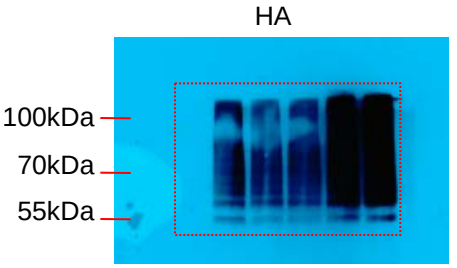

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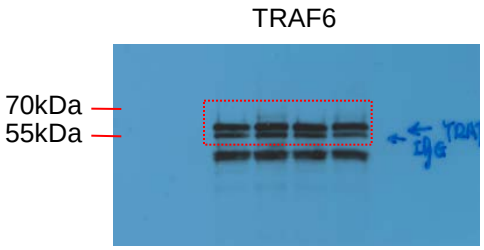

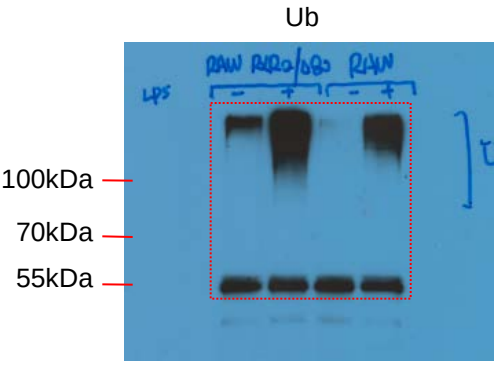

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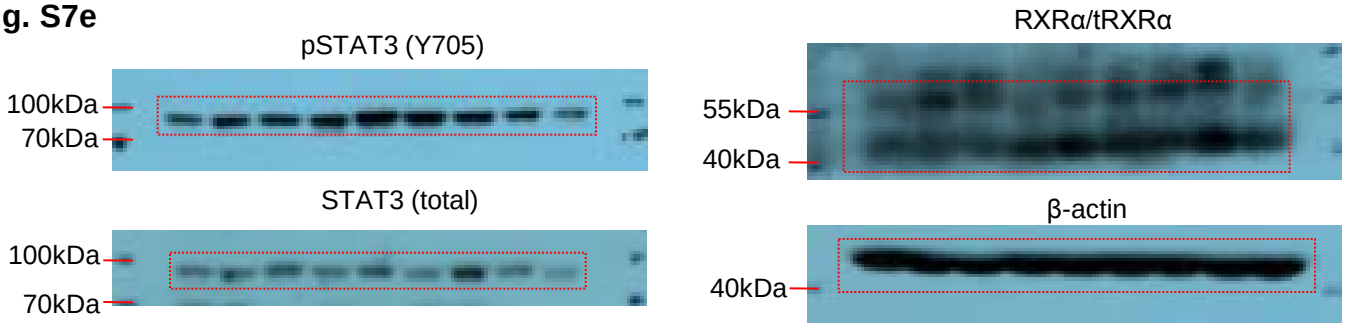

Fig. S7g

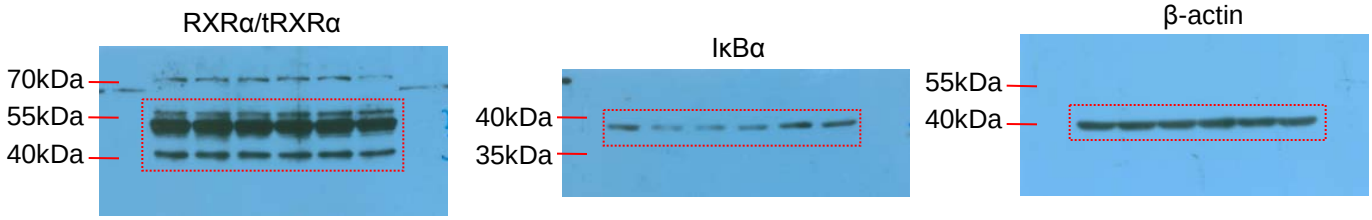

Fig. S7j

Supplementary Figure 8. Unprocessed scans of immunoblots shown in the main figures.