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Supplemental Material to:

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Small molecule-driven mitophagy-mediated NLRP3 inflammasome inhibition is responsible for the prevention of colitis-associated cancer

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The primer sequences used in this study were as follows:

Tnf forward 5’-CGAGTGACAAGCCTGTAGCCC-3’;

Tnf reverse 5’-GTCTTTGAGATCCATGCCGTTG-3;

Il1b forward 5’-CTTCAGGCAGGCAGTATCACTC-3’;

Il1b reverse 5’-TGCAGTTGTCTAATGGGAACGT-3’;

Il6 forward 5’-ACAACCACGGCCTTCCCTAC-3’;

Il6 reverse 5’-TCTCATTTCCACGATTTCCCAG-3’;

Il17a forward 5’-TCGAGAAGATGCTGGTGGGT-3’;

Il17a reverse 5’-CTCTGTTTAGGCTGCCTGGC-3’;

Ifng forward 5’-AGCAACAGCAAGGCGAAA-3’;

Ifng reverse 5’-CTGGACCTGTGGGTTGTTGA-3’;

Hif1a forward 5’-AGCTTCTGTTATGAGGCTCACC-3’;

Hif1a reverse 5’-TGACTTGATGTTCATCGTCCTC-3’;

Ptgs2 forward 5’-ACACTCTATCACTGGCATCC-3’;

Ptgs2 reverse 5’-GAAGGGACACCCTTTCACAT-3’;

Actb forward 5’-TGCTGTCCCTGTATGCCTCT-3’;

Actb reverse 5’-TTTGATGTCACGCACGATTT-3’.

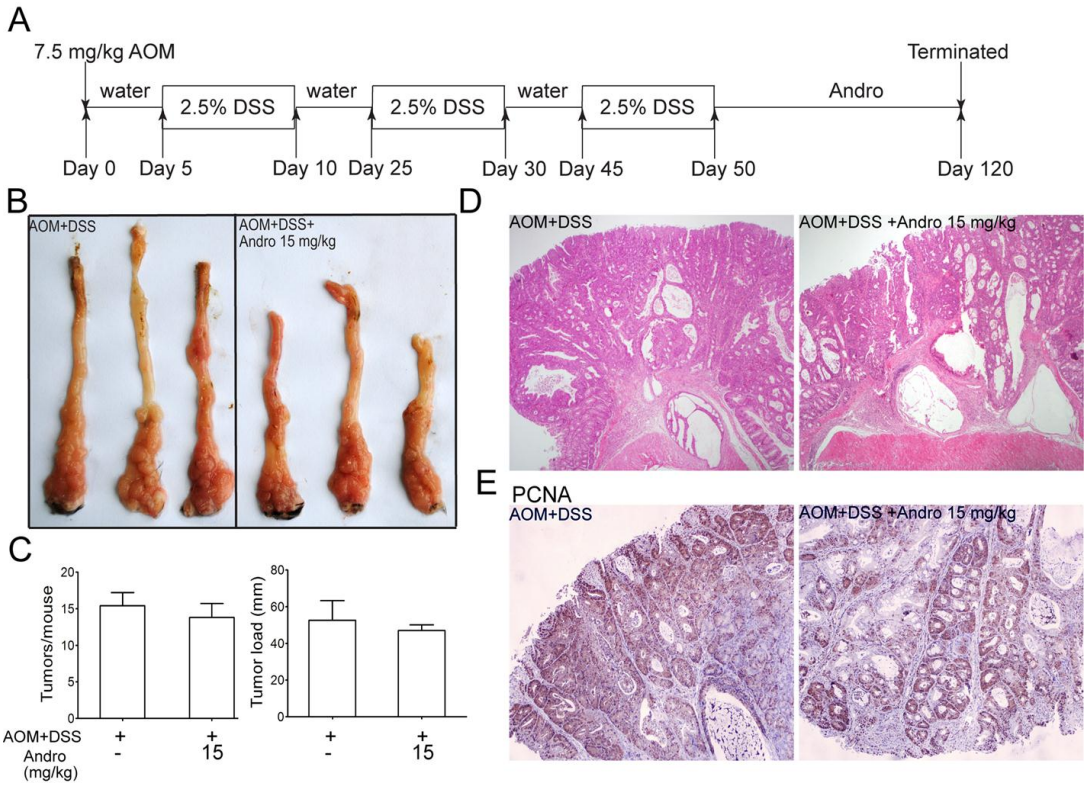


Figure S1. Andrographolide given to mice after a tumor has been formed showed a minor effect on inhibiting tumor growth in a colitis-associated cancer model. Mice were subjected to an azoxymethane (AOM)-based colitis-associated cancer (CAC) induction protocol using 3 cycles of 2.5% dextran sulfate sodium (DSS) in the drinking water. (**A**) The flowsheet of the AOM-DSS model. Andrographolide (Andro) was i.g. (intragastrically) given daily from day 50 to day 120. Mice were scarified at day 120 after CAC induction. (**B**) The inside of the colon was photographed. (**C**) Tumor load was determined by summing all tumor diameters for a given animal. (**D**) Colon tissues were fixed and stained with H&E. (**E**) PCNA expression was analyzed by immunochemistry on paraffin-embedded colon sections. Data represent means ± SEM of 8 mice.

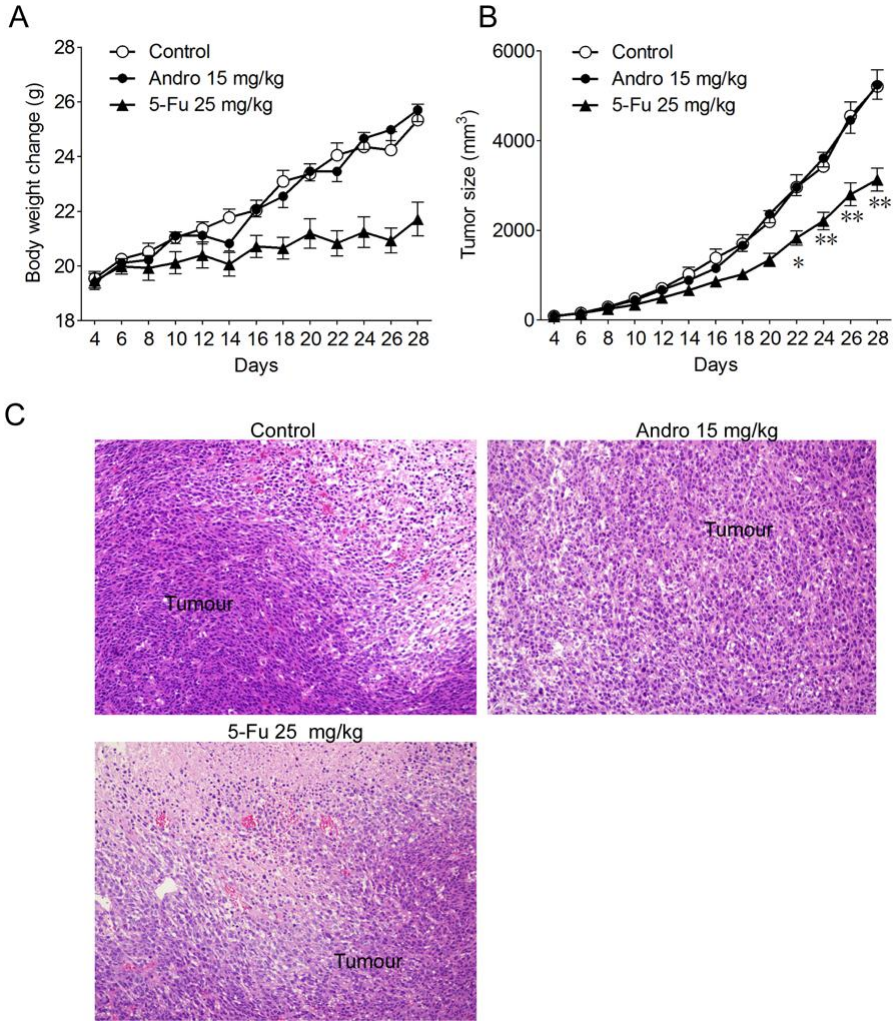


Figure S2. Andrographolide at the dose of 15 mg/kg did not inhibit mouse colon carcinoma CT26 cell growth. 1×10^6 CT26 cells were injected subcutaneously into the right flank of BALB/C mice. Four days after injection, the tumor-bearing mice were distributed into 3 groups. Next, they were treated with 15 mg/kg andrographolide (Andro, once a day), 25 mg/kg 5-fluorouracil (5-Fu, once every 4 days) or PBS for a further 24 days, respectively. (A) Body weight change of the mice in each group. (B) Time course of tumor growth. Data are mean ± SEM of 15 mice in each group. *P<0.05, **P<0.01 vs. Control. (C) At day 28, tumors in each group were removed. Tumor sample sections from each group were stained with H&E.

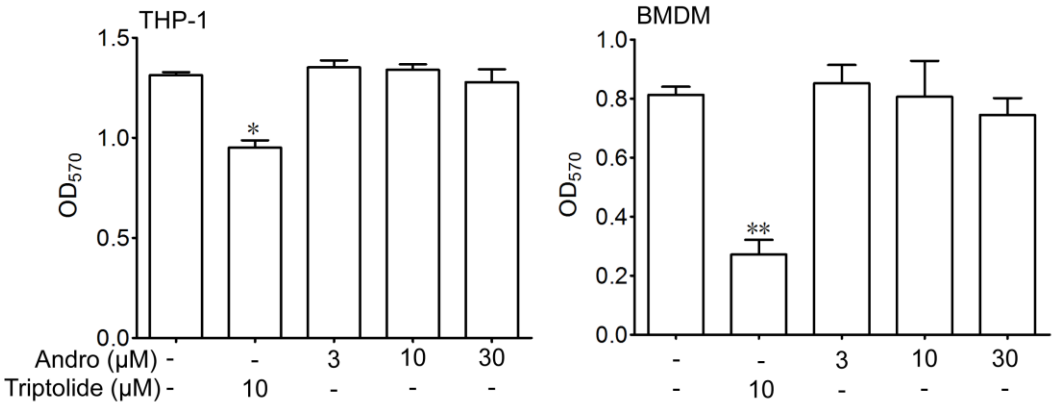


Figure S3. Andrographolide did not influence the survival of macrophages in vitro. Human monocytic THP-1 cells and murine bone marrow-derived macrophage (BMDM) cells were incubated with andrographolide (Andro) and triptolide for 12 h and the MTT assay was used to examine cell viability. The results were expressed as mean ± SEM of 3 experiments. **P*<0.05, ***P*<0.01 vs. drug-untreated group.

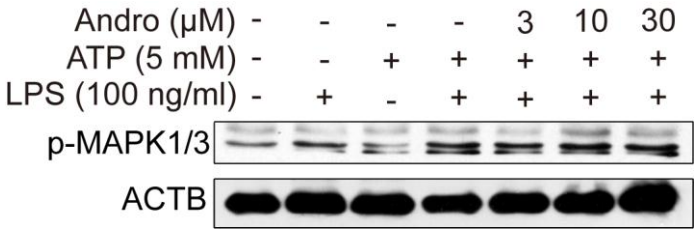


Figure S4. A MAPK1/3-mediated pathway was not involved in andrographolide (Andro)-induced autophagy. Human monocytic THP-1 cells were cultured with 100 ng/ml LPS for 3 h, then cells were treated with Andro (3, 10, 30 μM) for 1 h, followed by 1 h incubation of 5 mM ATP. Proteins were collected and phosphorylation of MAPK1/3 was detected by western blot. Data shown are representative of 3 experiments.

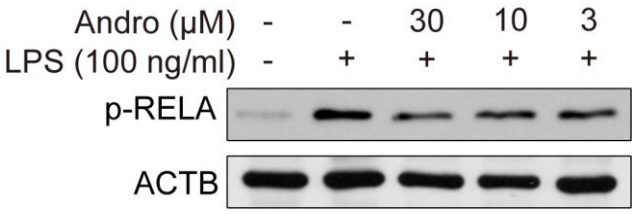


Figure S5. Andrographolide (Andro) inhibited NFKB activation in a concentration-dependent manner. Human monocytic THP-1 cells were cultured with 100 ng/ml LPS for 3 h, then cells were treated with Andro (3, 10, 30 μM) for 1 h. Proteins were collected and phosphorylation of RELA/NFKB p65 was detected by western blot. The data shown here are representative of 3 different experiments.

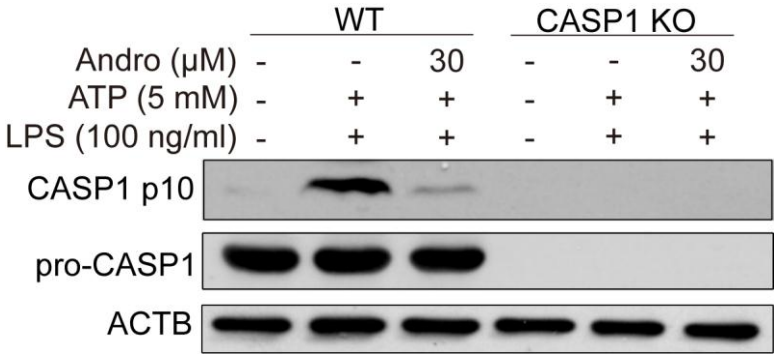


Figure S6. Western blot for CASP1 in macrophage from wild type and CASP1/caspase 1 knockout mice. Macrophages were isolated from spleen of wild type and CASP1 knockout mice using commercial magnetic beads (Miltenyi) as described in Materials and Methods. Then cells were cultured with 100 ng/ml LPS for 3 h, then cells were treated with andrographolide (Andro) (3, 10, 30 μM) for 1 h, followed by 1 h incubation with 5 mM ATP. Proteins were collected and CASP1 activation was detected by western blot. CASP1 knockout mice (NOD.129S2(B6)-Casp1^{tm1Sesh}/LtJ) were a gift from Professor Gang Hu (Nanjing Medical University, Nanjing).

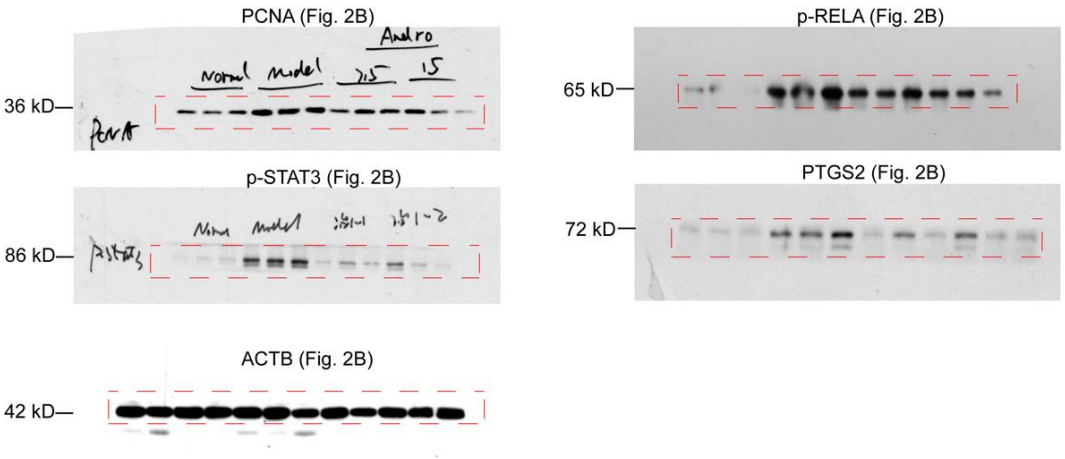
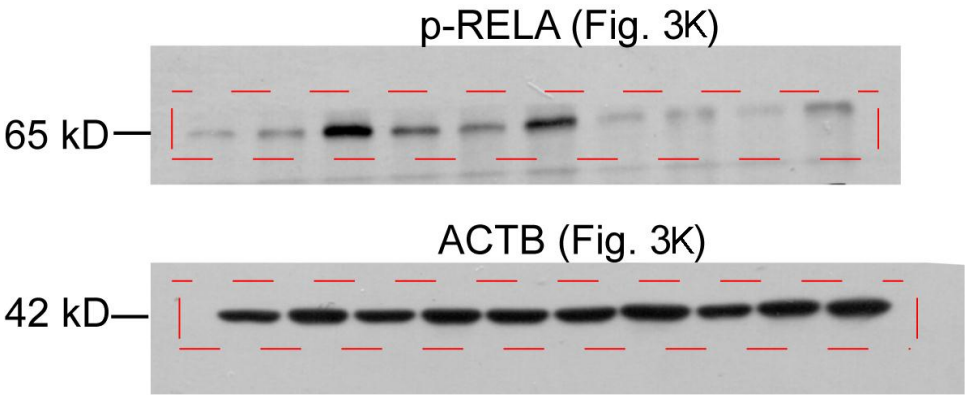


Figure S7. Complete western blotting gels from Figure 2.



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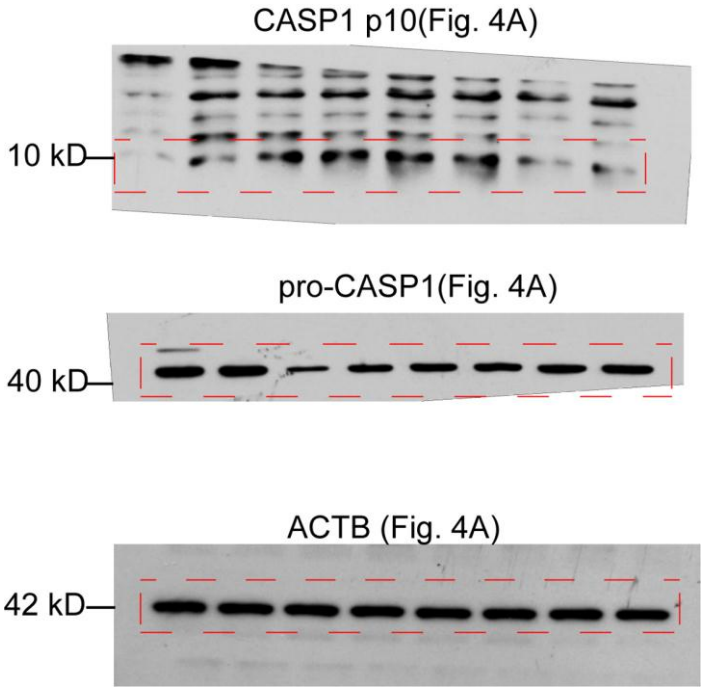

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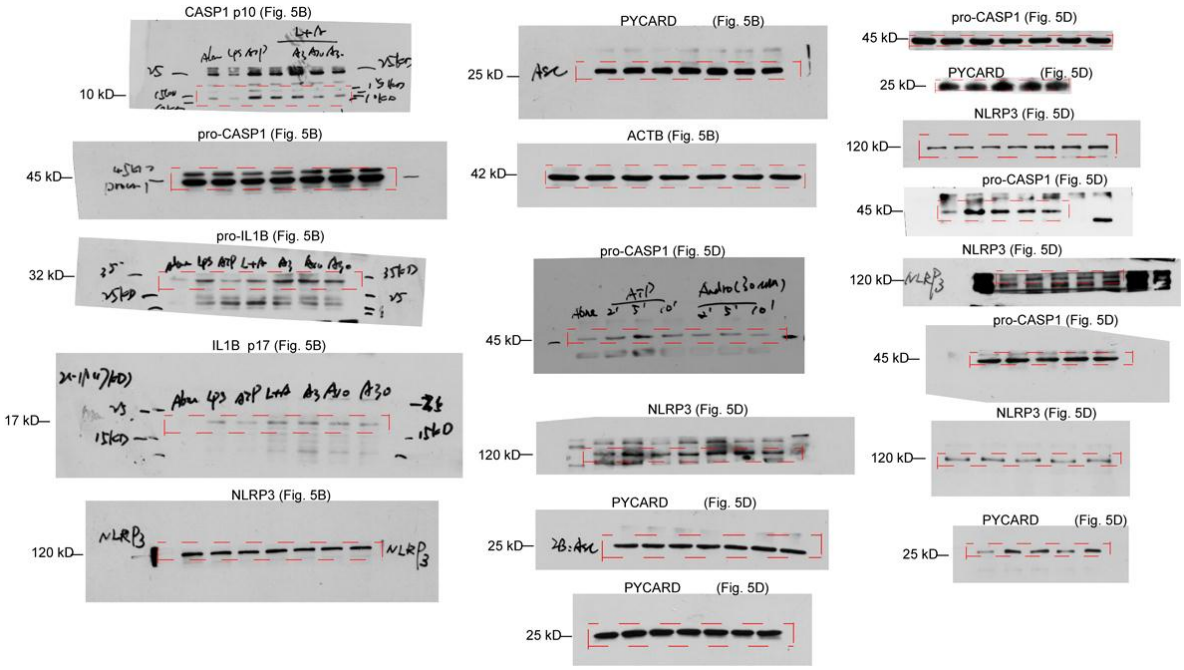

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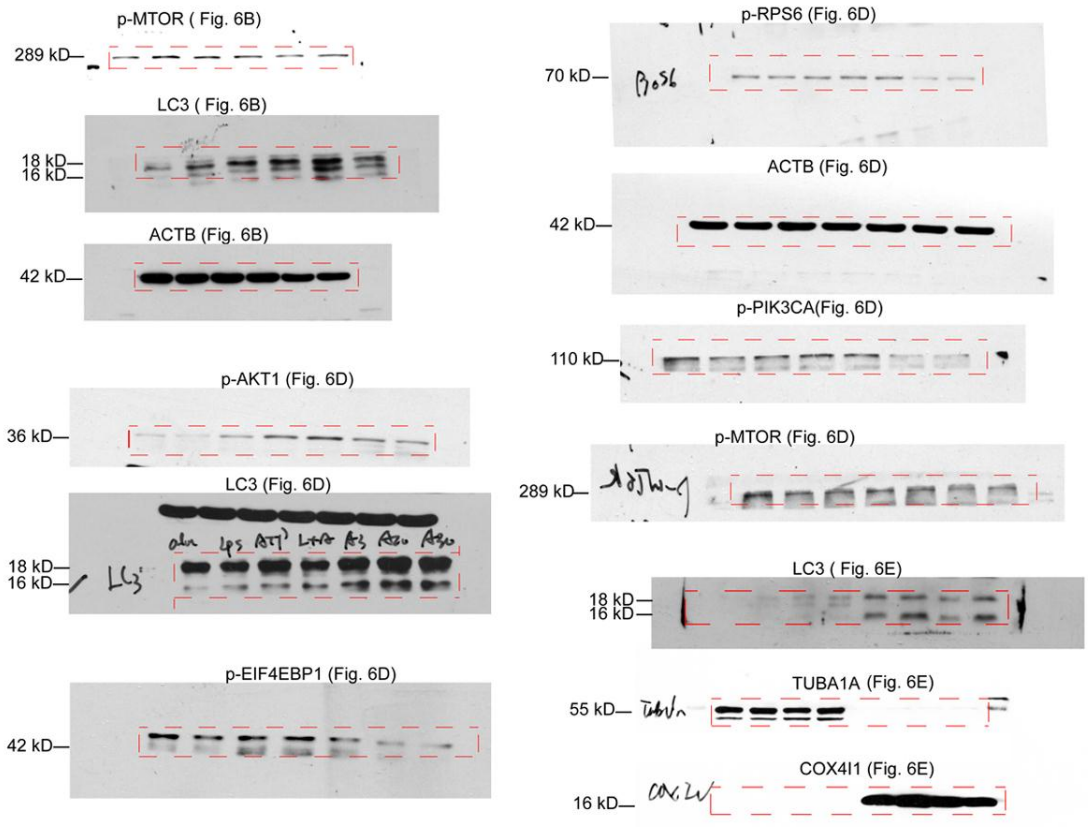

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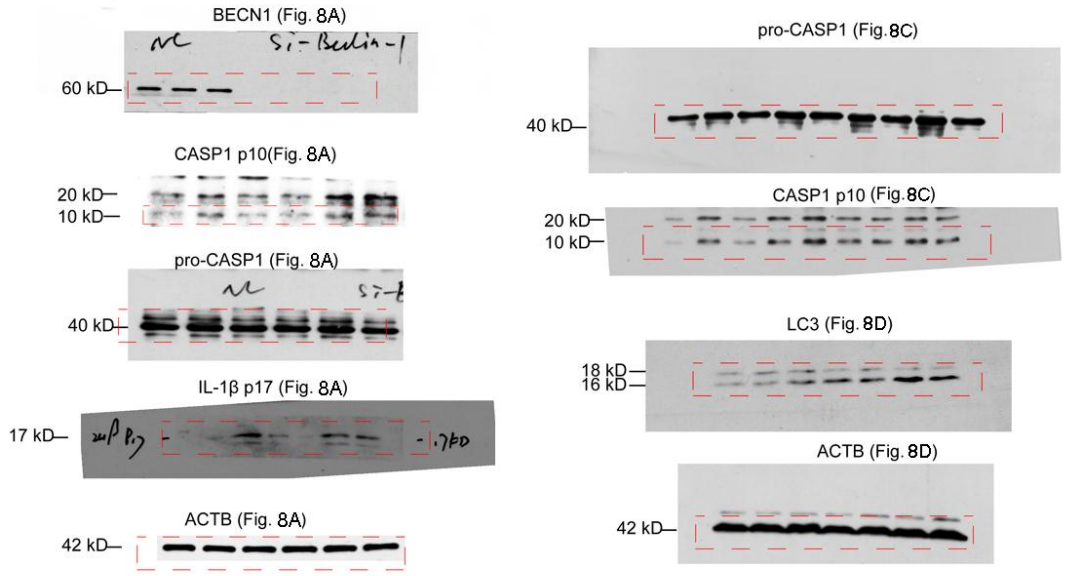

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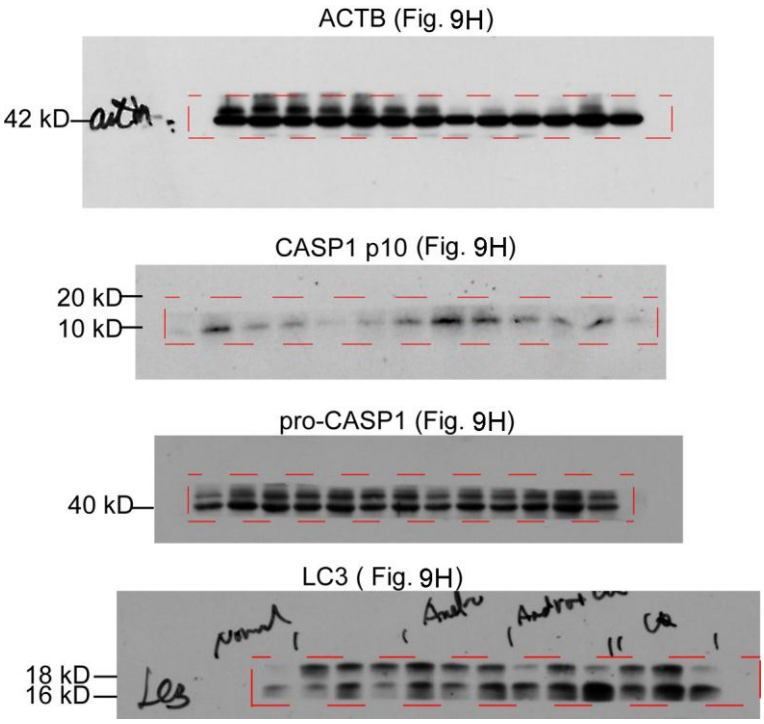

Figure S13. Complete western blotting gels from Figure 9.