Supplemental methods

Cell culture and drug treatment. HT-29 colon cancer cells were obtained from ATCC and maintained at 37°C in 5% CO_2 in Mccoy's 5A medium(Invitrogen, Carlsbad, CA) supplemented with 10% FBS (HyClone, Logan, UT), 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen) as described (1, 2). The cells were exposed to 150 nM Smac mimetic GT-A, and the control compound, GT-C (3) with or without human TNF-α neutralizing antibody (R&D system, Minneapolis, MN, USA) (5μg/ml) for 48 h. Cells were analyzed for apoptosis using nuclear fragmentation assay.

Reverse transcriptase PCR (RT–PCR) and real-time PCR analysis. Total RNA was isolated from HNSCC cells using the RNAgents Total RNA Isolation System (Promega, Madison, WI, USA). First-strand cDNA was synthesized from 5 μg of total RNA using Superscript II reverse transcriptase (Invitrogen). The primers used for cIAP1 are 5-TGTCAGCACTTCTTAATGCTG-3 and 5- CAGTTCTCTCGCTTGTAAAGG-3, for cIAP2 are 5- AGCCAGTTACCCTCATCTAC-3 and CTACTAAAGCCCATTTCCAC-3, and for GAPDH are 5-CTCAGACACCATGGGGAAGGTGA-3 and 5-ATGATCTTGAGGCTGTTGTCATA-3. The detailed conditions for real-time PCR are available on request (4-6).

Supplemental references

1. Yu J, Zhang L, Hwang PM, Rago C, Kinzler KW, Vogelstein B. Identification and classification of p53-regulated genes. Proc Natl Acad Sci U S A 1999;96: 14517-22.

2. Kohli M, Yu J, Seaman C, *et al.* SMAC/Diablo-dependent apoptosis induced by nonsteroidal antiinflammatory drugs (NSAIDs) in colon cancer cells. Proc Natl Acad Sci U S A 2004;101: 16897-902.

3. Bank A, Wang P, Du C, Yu J, Zhang L. SMAC mimetics sensitize nonsteroidal anti-inflammatory drug-induced apoptosis by promoting caspase-3-mediated cytochrome c release. Cancer Res 2008;68: 276-84.

4. Sun Q, Ming L, Thomas SM, *et al.* PUMA mediates EGFR tyrosine kinase inhibitor-induced apoptosis in head and neck cancer cells. Oncogene 2009;18: 2348-57.

5. Wu B, Qiu W, Wang P, *et al.* p53 independent induction of PUMA mediates intestinal apoptosis in response to ischaemia-reperfusion. Gut 2007;56: 645-54.

6. Qiu W, Carson-Walter EB, Liu H, *et al.* PUMA regulates intestinal progenitor cell radiosensitivity and gastrointestinal syndrome. Cell Stem Cell 2008;2: 576-83.

Supplemental figure legends

Figure S1. Smac mediates apoptosis induced by gemcitabine in HNSCC cells. Apoptosis was analyzed by flow cytometry to quantitate Annexin V-positive cells. P- parental cells; KD1, KD2- two independent knockdown clones.

Figure S2. The Bcl-2 family of proteins in gemcitabine- and Smac-induced apoptosis. **(A)** JHU-012 and 1483 cells treated with 50 μM gemcitabine for 48 h. The indicated BH3-only proteinsand p53 were analyzed by Western blotting. **(B)** *Upper*, the indicated

HNSCC lines were infected with PUMA adenovirus (Ad-PUMA) or the BH3 domain deleted PUMA adenovirus (Ad-ΔBH3) for 48 h. Apoptosis was determined by nuclear fragmentation assay. *Lower*, the indicated parental (P) or *SMAC* knockdown (KD) HNSCC cells were transfected with Noxa or empty vector (pcDNA3.1) for 48 h. Apoptosis was analyzed by nuclear fragmentation assay. **, $P < 0.01$, *, $P < 0.05$, KD *vs.* P. **(C)** BH3-only proteins sensitized HNSCC cells to apoptosis induced by gemcitabine. JHU-012 and 1483 cells were treated with 10 μM gemcitabine with or without Ad-PUMA or transfected Noxa for 48 h. Apoptosis was determined by nuclear fragmentation assay. **, $P < 0.01$, *, $P < 0.05$, combination *vs.* single agent.

Figure S3. The Smac mimetic sensitizes HNSCC cells to gemcitabine through apoptosis. **(A)** Cytochrome *c* release to the cytosol was analyzed by Western blotting in HNSCC cells with indicated treatment for 48 h. **(B)** Smac mimetic synergized with gemcitabine to suppress clonogenic survival of HNSCC cells. Cells plated at 6 h following by 10 μM gemcitabine or left untreated, and long-term cell growth was assessed by colony formation assay. Representative pictures of colonies are shown. **(C)** Quantitation of colony numbers. **, $P < 0.01$, GT-A+Gem *vs.* GT-C+Gem.

Figure S4. The Smac mimetic sensitizes HNSCC cells to cisplatin. **(A)** The indicated HNSCC cells were treated with 50 μM cisplatin with or without 100 nM control (GT-C) or active (GT-A) Smac mimetic. Apoptosis was determined by nuclear fragmentation assay at 48 h. **, $P < 0.01$, GT-A+Cis *vs.* GT-C+Cis. **(B)** JHU-022 cells were treated as in (A) and analyzed for apoptosis at indicated time points. **, $P < 0.01$, GT-A+Cis *vs.*

GT-C+Cis. **(C)** The indicated HNSCC cells were treated with 10 μM cisplatin with or without 100 nM GT-A for 6 h, then plated at 1:500 dilution (~400 cells per well) in 12-well plates and allowed to form colonies for 14 days. Number of colonies containing >50 cells were scored and the relative clonogenic survival was calculated with untreated cells set at 100%. **, $P < 0.01$, combination *vs.* single agent.

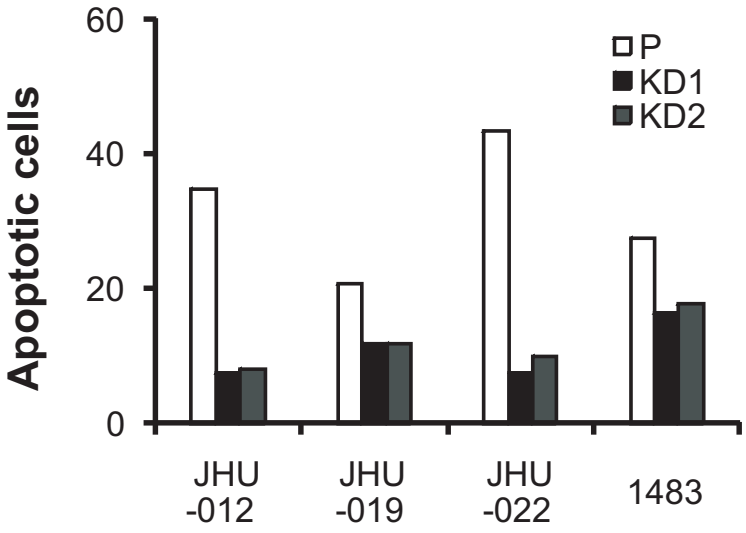
Figure S5. The Smac mimetic did not decrease *cIAP-1/2* mRNA levels. JHU-012 and JHU-022 cells were subjected to GT-A (100 ng/ml) for indicated times. The levels of *cIAP-1/2* mRNA were determined by real-time PCR with values normalized to *GAPDH*.

Figure S6. The Smac mimetic activated and synergized with TNF-α signaling in cancer cells. **(A)** The JHU-012 and JHU-019 cells were preincubated with TNFα antibody (5 μg/ml) for 1 h, then treated with TNFα (100 ng/ml) alone or combined with GT-A (100 nM) for 48 h. Apoptosis was analyzed by nuclear fragmentation assay. **, $P < 0.01$, *, $P < 0.05$, TNF-α+Ab *vs.* TNF-α (bar 3 *vs.* 2); TNF-α+GT-A *vs.* TNF-α (bar 4 *vs.* 2); TNF-α+GT-A+Ab *vs.* TNF-α+GT-A (bar 5 *vs.* 4). **(B)** HT-29 colon cancer cells were subjected to GT-A (150 nM) for 48 h. Apoptosis was analyzed by nuclear fragmentation assay. **, $P < 0.01$, GT-A *vs.* Un (bar 3 *vs.* 1); GT-A *vs.* GT-A+Ab (bar 3 *vs.* 4).

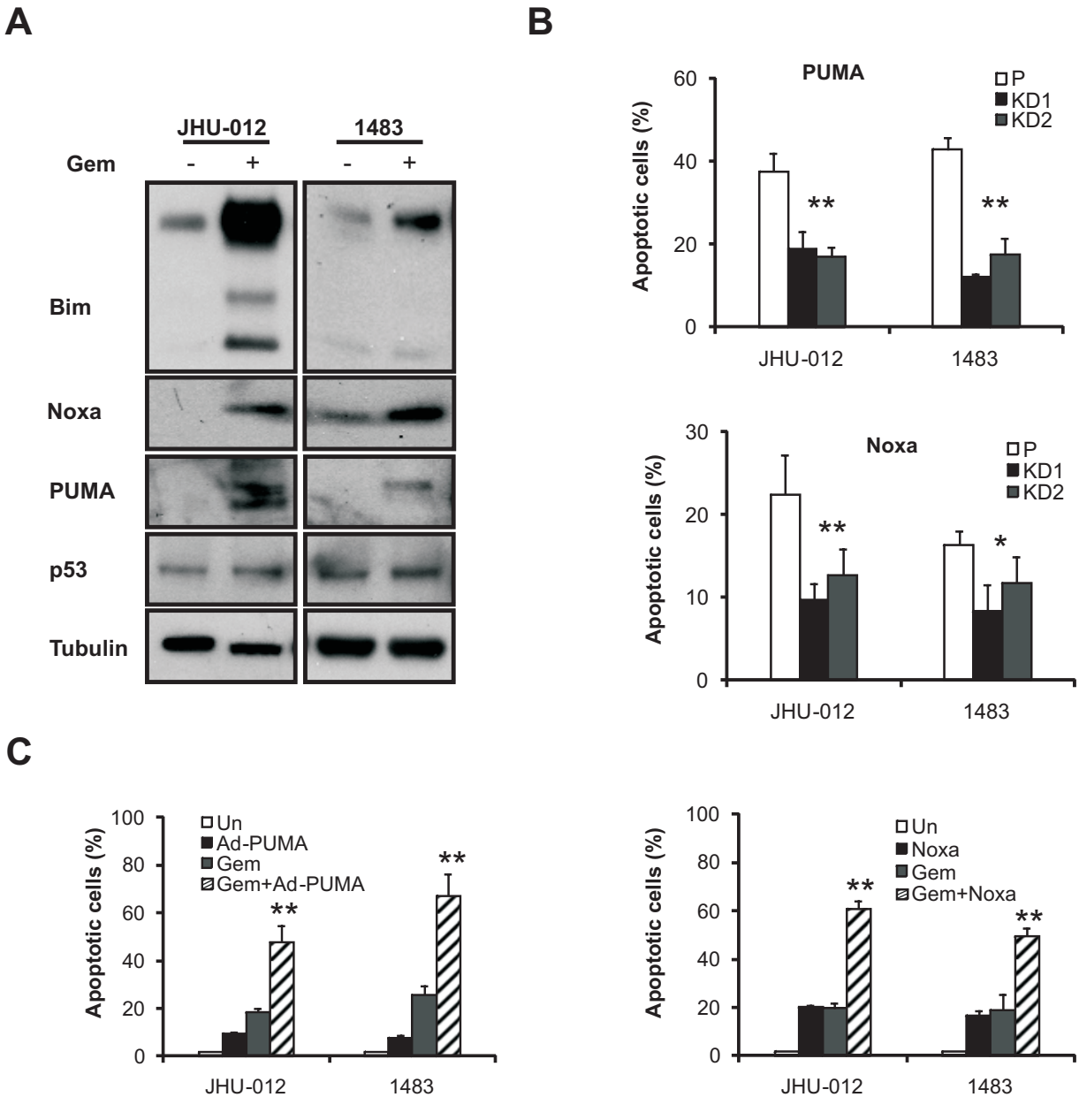
Figure S7. Smac modulates the therapeutic responses to gemcitabine in HNSCC xenografts. (A) Growth curve of JHU-012 parental (P) and *SMAC* knockdown (KD) tumors **(**n = 7 per group) subjected to indicated treatments. Three treatments of

gemcitabine on day 10, 13 and 16 are indicated by arrows**.** **, $P < 0.01$. KD1+Gem *vs.* P+Gem, *, P<0.05. KD1 + Gem *vs.* KD1 + ddH_2O**.** **(B)** Index of TUNEL-positive or BrdU-labeled cells in JHU-012 tumors 24 h after the second gemcitabine injection. **, $P < 0.001$, KD1 + Gem *vs.* P + Gem.

Sun et al. Figure S1



Sun et al. Figure S2



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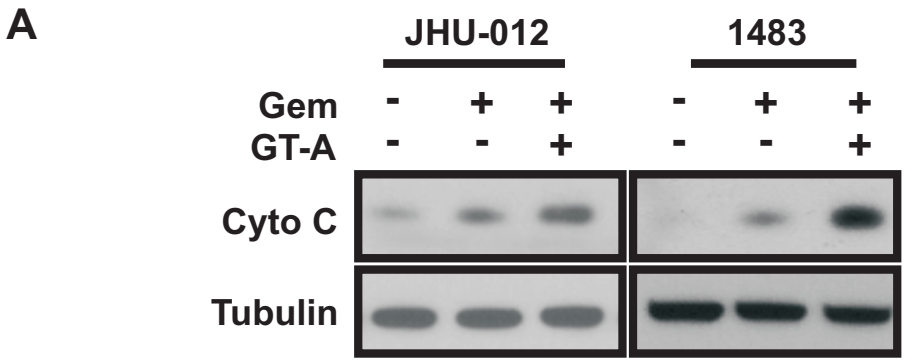

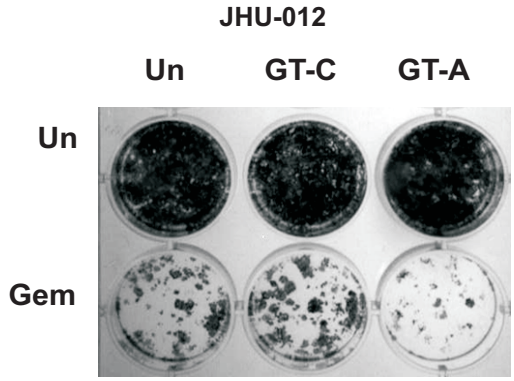

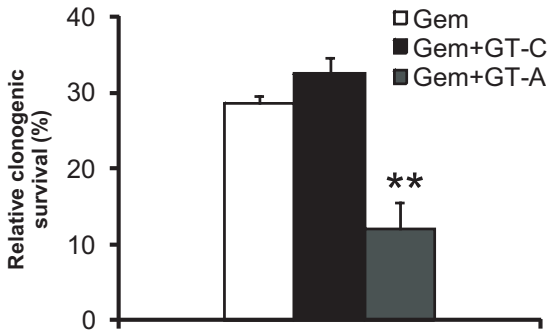

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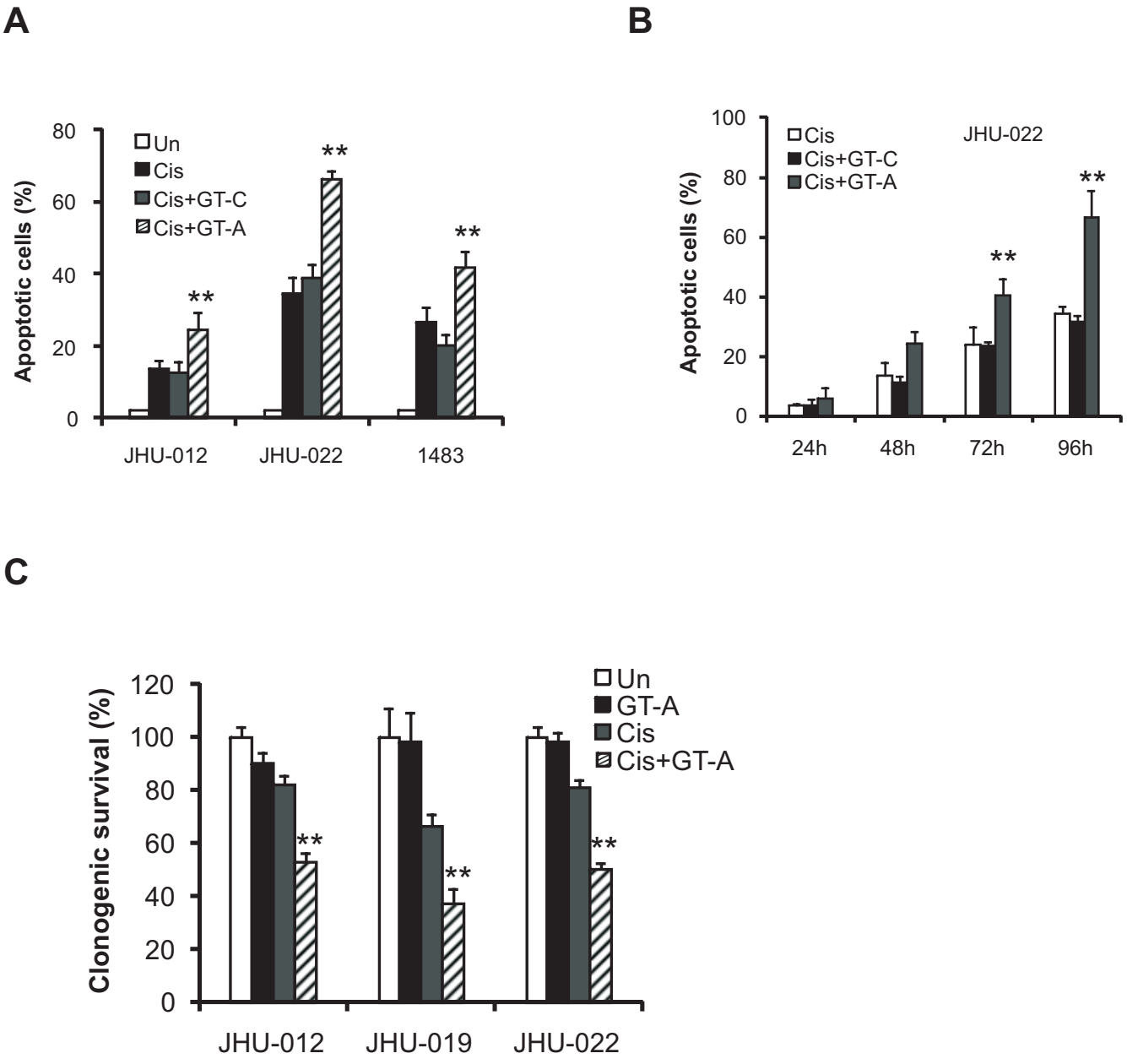

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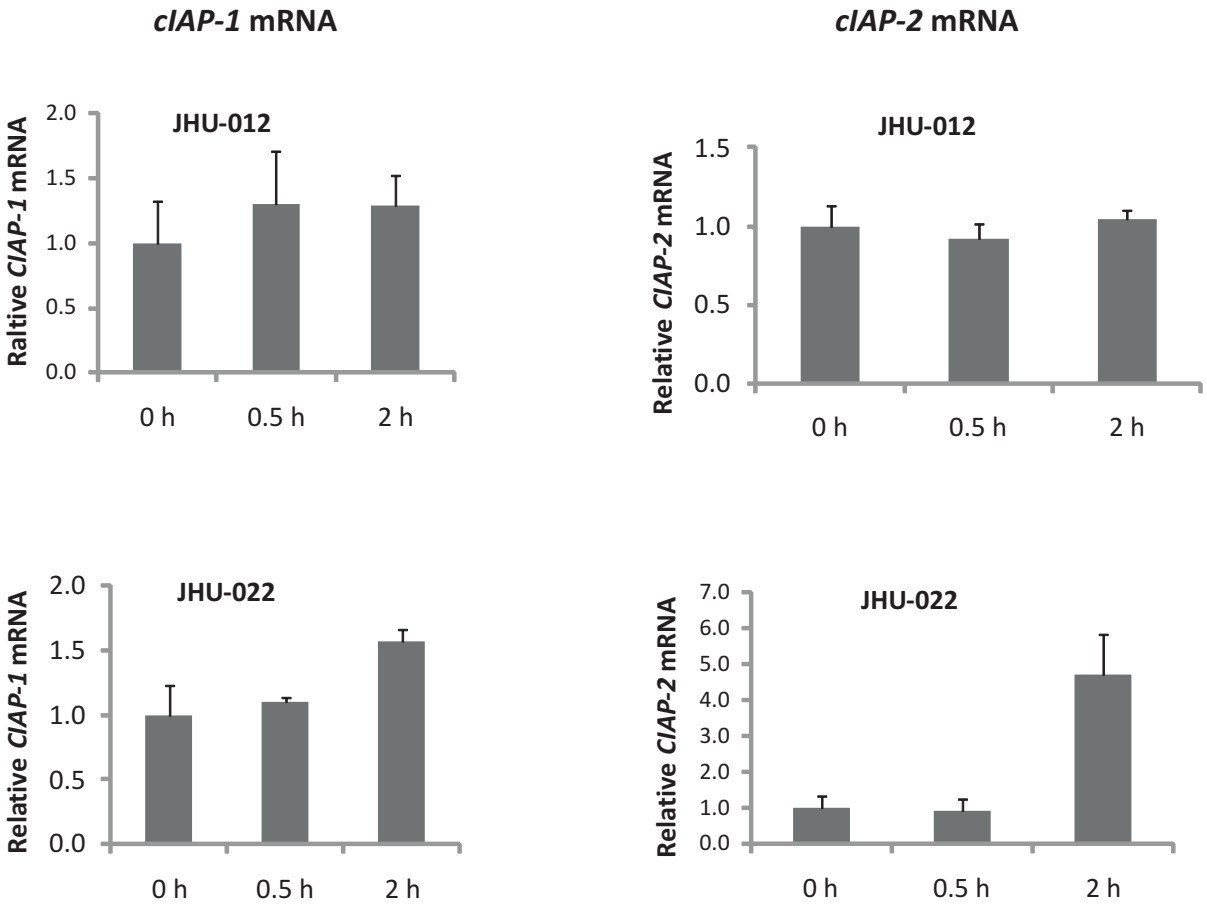

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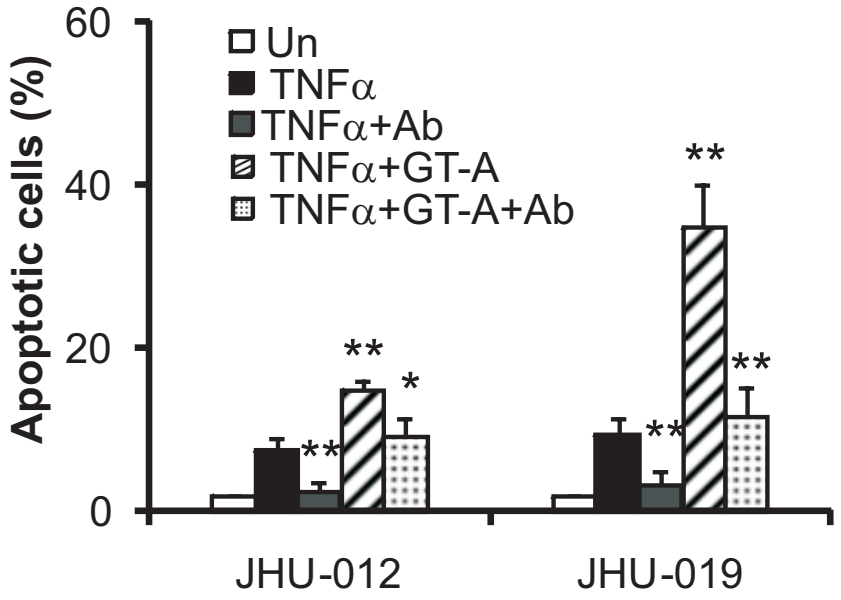
A



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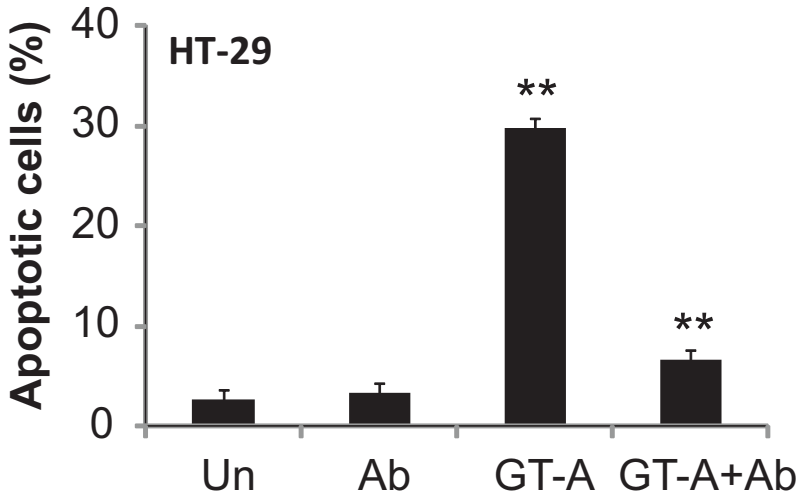

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