Supporting Information

Leibowitz et al. 10.1073/pnas.1415178111

SI Materials and Methods

Immunohistochemistry and Immunofluorescence. Tissue sections (5 μm) were deparaffinized, rehydrated through graded ethanols, and treated with 3% (wt/vol) hydrogen peroxide (IHC only), followed by antigen retrieval for 10 min in boiling 0.1 M citrate buffer (pH 6.0) with 1 mM EDTA. Apoptosis was analyzed by TUNEL staining with the ApopTag Fluorescein In Situ Apoptosis Detection Kit (Chemicon International) according to the manufacturer's instructions. For active caspase 8 staining, sections were prepared as above, and nonspecific antibody binding was blocked by using 50% (vol/vol) goat serum at room temperature for 45 min. Sections were incubated overnight in a humidified chamber at 4 °C with rabbit anticleaved caspase-8 (NB100-56116; 1:600; Novus Biologicals). Sections were then incubated for 1 h at room temperature with AlexaFluor 594-conjugated goat anti-rabbit secondary antibodies (A11012; 1:250, Invitrogen) and mounted with VectaShield + DAPI (Vector Labs). For active caspase 8/OLFM4 double staining, sections were stained for active caspase 8 as described. Following secondary antibody incubation, sections were washed in PBS and incubated overnight at 4 °C with mouse anti-OLFM4 (Clone N212; 1:50, a gift from Wataru Yasui in Hiroshima University, Hiroshima, Japan). Sections were then incubated for 1 h at room temperature with AlexaFluor 488-conjugated goat anti-mouse secondary antibodies (A11001; 1:250, Invitrogen), and mounted with VectaShield + DAPI.

Human Tissue Lysate Preparation. For human adenoma tissues, ∼30 mg of tissue was collected in homogenization buffer (0.25 M sucrose, 10 mM Hepes, and 1 mM EGTA) supplemented with protease inhibitors (Complete mini, EDTA-free; Roche) and homogenized with 25 strokes in a Dounce homogenizer by using a "loose" pestle, followed by 25 strokes with a "tight" pestle. Samples were then cleared by two rounds of centrifugation at 1,000 × *g* for 10 min at 4 °C. Cleared lysate was assayed for protein concentration and stored at −80 °C.

Western Blotting. Cell lysate preparation and immunoblotting were performed as described (1). The antibodies used for Western blotting included those for human BID, caspase 7, caspase 8, caspase 9, DR5 (Cell Signaling Technologies), β-actin (Sigma), BAK, DR4, SMAC/Diablo (EMD Millipore), caspase 3 (Enzo Life Sciences), cleaved caspase 3, cytochrome *c* (BD Pharmingen), COX IV (Invitrogen), Bax, FADD (BD Transduction Labs), and α-tubulin (Oncogene Sciences). All Western blots are representative of at least two independent experiments.

Mice. Mice were housed in microisolator cages in a room illuminated from 0700–1900 hours (12:12-h light-dark cycle), with access to water and chow ad libitum. Female C57BL/6J $BID^{-/-}$ (*BID* KO) mice, which were described (2), were crossed with male $APC^{Min/+}$ mice (Jackson Laboratory) to generate $APC^{Min/+}/BID^{+/-}$ male mice, which were then crossed to $BID^{+/-}$ females to generate $APC^{Min/+}$ littermates with homozygous WT ($^{+/+}$), heterozygous ($^{+/-}$), or null ($^{-/-}$) *BID* alleles. For analysis of intestinal stem cell apoptosis, the described *Lgr5-EGFP* (Lgr5-EGFP-IRES-creERT2) mice (3) were crossed with *BID* KO mice to generate *Lgr5-EGFP*–marked $BID^{+/-}/APC^{Min/+}$ male mice, which were crossed with $BID^{+/-}$ female mice to generate *Lgr5-EGFP*–marked $BID^{+/+}$ and $BID^{-/-}$ $APC^{Min/+}$ mice. Genotyping of *APC* alleles was performed according to the Jackson Laboratory protocol, and genotyping of *BID* and *Lgr5-EGFP* alleles was performed as described (2, 3).

Tissue Preparation and Analysis of Adenomas, Histology, and Apoptosis in Mice. Following NSAID treatment and killing of mice, intestinal tracts were dissected and fixed in 10% (vol/vol) formalin for histological analysis. Adenomas were counted under a dissecting microscope. Histological analysis was performed by hematoxylin and eosin (H&E) staining. Apoptosis was analyzed by TUNEL staining as described above. Cells were counted in complete crypts extending to neighboring villi and containing at least 17 cells along either side with several Paneth cells at the bottom. TUNEL-positive cells were scored in 100 crypts per tissue segment, with a minimum of three mice per group. Active caspase 3 and Lgr5 (GFP)/TUNEL double staining was performed as described (4). Active caspase 8 staining was performed as described above.

Prostaglandin Metabolite Analysis. Five-week-old $BID^{+/+}$ and $BID^{-/-}$ $APC^{Min/+}$ mice were treated for 1 wk with AIN-93G diet containing sulindac or indomethacin. Blood was collected from the right ventricle of the treated mice in Microtainer Plasma Separator Tubes with Lithium Heparin (BD Biosciences). After centrifugation at 13,000 × *g* for 3 min, plasma samples were stored at −80 °C until assayed. Prostaglandin E2 metabolites (PGEM) were measured by using the Prostaglandin E Metabolite EIA kit (Cayman) according to the manufacturer's instructions.

***OLFM4* RNA in Situ Hybridization/TUNEL Double Staining.** Probe template containing a 705-bp region of *OLFM4* was cloned into pBluescript SK^+, modified to contain the T7 promoter (5). Linearized plasmid was used as template to generate digoxigenin (DIG)-labeled RNA probes by using the DIG RNA Labeling Kit (SP6/T7) (Roche). Tissue sections (5 μm) were rehydrated, denatured with 0.2 M HCl, treated with Proteinase K, postfixed in 4% (wt/vol) paraformaldehyde, and acetylated. After prehybridization for 1 h at 57 °C, denatured DIG-labeled probes were hybridized to sections at 57 °C for 16–24 h. Hybridization buffer contained 65% (vol/vol) formamide, 5× SSC, 2% (wt/vol) Roche blocking powder, 1 μg/mL yeast tRNA, 50 μg/mL heparin, 5 mM EDTA, and 0.05% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate. After washing, slides were incubated with DIG-horseradish peroxidase (Abcam), biotinyl tyramide (DAKO), biotin-horseradish peroxidase (Abcam), and then additional biotinyl tyramide to amplify the signal. Slides were then incubated with streptavidin-conjugated AlexaFluor 594 followed by a PBS wash. TUNEL staining was performed with the ApoAlert DNA Fragmentation Assay Kit (Clontech) according to manufacturer's instructions, with minor modifications. Briefly, slides were incubated for 10 min with equilibration buffer, and then incubated for 1 h at 37 °C in TdT incubation buffer (equilibration buffer, TdT enzyme, fluorescent nucleotide mix). The reaction was halted by a 15-min incubation in 2× SSC stop buffer. Slides were washed in PBS and mounted with VectaShield + DAPI (Vector Labs) for visualization.

Cell Culture and Drug Treatment. Human CRC cell lines, including HCT116, HT29, RKO, DLD1 (American Type Culture Collection), and their derivatives, were cultured in McCoy's 5A media (Invitrogen). NCM356 cells (Incell) were described (6) and cultured in M3 media (Incell) according the supplier's instructions. Cells were maintained at 37 °C and 5% (vol/vol) CO_2,

and cell culture media were supplemented with 10% (vol/vol) defined FBS (HyClone), 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). For drug treatment, cells were plated in 12-well plates at ∼30% density 24 h before treatment. Stock solutions of sulindac sulfide (Merck), indomethacin (Sigma), camptothecin (CPT) (Sigma), staurosporine (STS) (EMD Biosciences), TRAIL (R&D Systems), sc-236 (Sigma), and sulindac sulfone (Sigma) were diluted to appropriate concentrations in culture media, and then added to cells for the treatment.

Targeting *BID* in HCT116 Cells. Gene targeting vectors were constructed by using the recombinant adeno-associated virus (rAAV) system as described (7). Briefly, two homologous arms (1.321 and 1.371 kb, respectively) flanking exon 4 and spanning exon 5 of *BID*, along with a neomycin-resistant gene cassette (*Neo*), were inserted between two Not I sites in the AAV shuttle vector pAAV-MCS (Stratagene). Packaging of rAAV was performed by using the AAV Helper-Free System (Stratagene) according to the manufacturer's instructions. HCT116 cells containing two copies of WT *BID* were infected with the rAAV and selected by G418 (0.5 mg/mL; Mediatech) for 3 wk. Drug-resistant clones were pooled and screened by PCR for targeting events with the following primers: P1 5′-TCCGCACGTCACTTCTTCCAA; P2 5′-TGGCAGTTGGGAGGATGTAGTTCT; NeoF 5′-TCTTGACGAGTTCTTCTGAG; and NeoR 5′-TTGTGCCCAGTCATAGCCG. Before targeting the second allele, the *Neo* cassette, which is flanked by Lox P sites, was excised from a heterozygous clone by infection with an adenovirus expressing Cre recombinase (Ad-Cre) (7). Single clones were screened by PCR for *Neo* excision, and a positive clone was infected again with the same *BID*-targeting construct. After the second round, *Neo* was again excised by Ad-Cre infection, and gene targeting in *BID* KO cells was verified by PCR and Western blotting.

Analysis of Apoptosis and Mitochondrial Membrane Integrity. After drug treatment, attached and floating cells were harvested and analyzed for apoptosis by counting cells with condensed chromatin and micronucleation after nuclear staining with Hoechst 33258 as described (1). A minimum of 300 cells were counted for each sample. Apoptosis was also analyzed by flow cytometry of cells stained with AlexaFluor 488-conjugated annexin V and propidium iodide (Invitrogen). All apoptosis measurements were repeated at least three times. The means + SDs are displayed in the figures. Bax oligomerization was analyzed by cross-linking the mitochondrial fractions with 1 mM dithiobis (DSP; Thermo Fisher) for 30 min, followed by SDS/PAGE under nonreducing conditions. To analyze mitochondrial membrane potential, NSAID-treated cells were incubated at 37 °C for 30 min with 100 nM MitoTracker Red CMX Rox (Invitrogen), and then analyzed by flow cytometry. Release of cytochrome *c* and SMAC/Diablo was assayed by Western blotting of mitochondrial and cytosolic fractions isolated from NSAID-treated cells as described (8). Long-term cell survival was assessed by colony formation assay as described (9). Cell viability was measured by using the CellTiter-Glo assay kit (Promega) according to the manufacturer's instructions.

Transfection and siRNA Knockdown. Full-length murine BID (pSFFV-BID) expression construct was described (10). All plasmids were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Small-interfering RNA (siRNA) duplexes (Dharmacon) include the following: BID-238, 5′- GAAGACAUCAUCCGGAAUA; DR5-215, 5′-AAGACCCUUGUGCUCGUUGUC; c-Myc-55, 5′-AACGUUAGCUUCACCAACAUU; APC-157, 5′-GGAAGUAUUGAAGAUGAAG; APC-8993, 5′-GCUGUGAAAUUCACAGUAAUA; cFLIP-226, 5′-AAGCAGUCUGUUCAAGGAGCA; and control scrambled siRNA. siRNA duplexes were transfected with Lipofectamine 2000 according to the manufacturer's instructions with minor modifications. Briefly, 200–400 pmols of siRNA duplexes were transfected into cells in 12-well plates for 4 h, followed by incubation in medium containing 5% (vol/vol) FBS for 20 h and then drug treatment in Complete media containing 10% (vol/vol) FBS.

Statistical Analysis. Multiple comparisons of the responses were analyzed by one-way ANOVA and Dunnett's post hoc test, whereas those between two groups were made by unpaired *t* test. Survival data were analyzed by log-rank test. All statistical tests were two-tailed.

1. Kohli M, et al. (2004) SMAC/Diablo-dependent apoptosis induced by nonsteroidal antiinflammatory drugs (NSAIDs) in colon cancer cells. *Proc Natl Acad Sci USA* 101(48): 16897–16902.
2. Yin XM, et al. (1999) Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. *Nature* 400(6747):886–891.
3. Barker N, et al. (2007) Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* 449(7165):1003–1007.
4. Qiu W, et al. (2010) Chemoprevention by nonsteroidal anti-inflammatory drugs eliminates oncogenic intestinal stem cells via SMAC-dependent apoptosis. *Proc Natl Acad Sci USA* 107(46):20027–20032.
5. van der Flier LG, Haegebarth A, Stange DE, van de Wetering M, Clevers H (2009) OLFM4 is a robust marker for stem cells in human intestine and marks a subset of colorectal cancer cells. *Gastroenterology* 137(1):15–17.
6. Zhang L, et al. (2010) Chemoprevention of colorectal cancer by targeting APC-deficient cells for apoptosis. *Nature* 464(7291):1058–1061.
7. Wang P, Yu J, Zhang L (2007) The nuclear function of p53 is required for PUMA-mediated apoptosis induced by DNA damage. *Proc Natl Acad Sci USA* 104(10): 4054–4059.
8. Bank A, Wang P, Du C, Yu J, Zhang L (2008) SMAC mimetics sensitize nonsteroidal anti-inflammatory drug-induced apoptosis by promoting caspase-3-mediated cytochrome c release. *Cancer Res* 68(1):276–284.
9. Zhang L, Yu J, Park BH, Kinzler KW, Vogelstein B (2000) Role of BAX in the apoptotic response to anticancer agents. *Science* 290(5493):989–992.
10. Kim TH, Zhao Y, Barber MJ, Kuharsky DK, Yin XM (2000) Bid-induced cytochrome c release is mediated by a pathway independent of mitochondrial permeability transition pore and Bax. *J Biol Chem* 275(50):39474–39481.

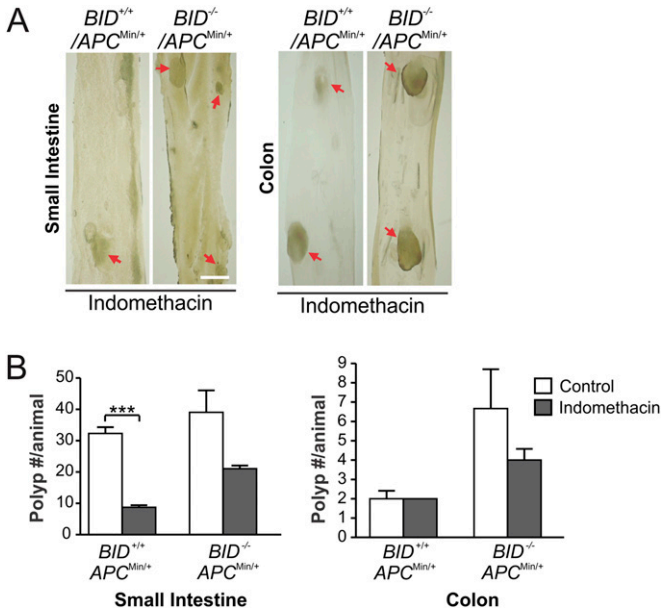

Fig. S1. The chemopreventive effect of indomethacin in WT and BID-deficient $APC^{Min/+}$ mice. (*A*) Representative pictures of the small intestine and colon from $BID^{+/+}$ and $BID^{-/-}$ $APC^{Min/+}$ mice fed with AIN93G diet ± indomethacin (10 ppm) for 2 mo, with arrows indicating example microscopic lesions. (Scale bar: 2 mm.) (*B*) Mean numbers + SD of small intestinal and colonic adenomas (≥0.5 mm in diameter) in $BID^{+/+}$ and $BID^{-/-}$ $APC^{Min/+}$ mice treated as in *A*. ***$P < 0.001$ ($n = 4$ per group).

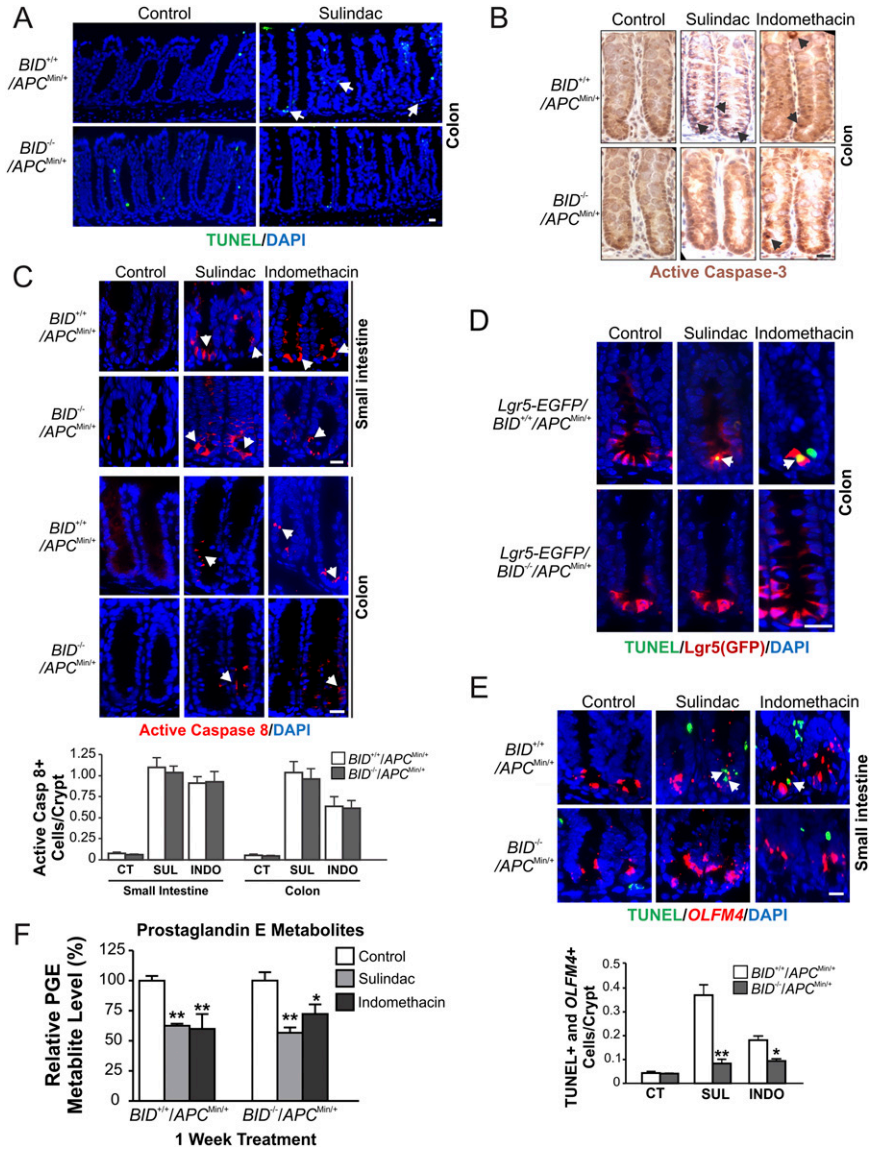

Fig. S2. Analysis of apoptosis and caspase activation in sulindac- or indomethacin-treated WT and BID-deficient $APC^{Min/+}$ mice. Mice with indicated genotypes were fed AIN93G diet ± sulindac (200 ppm) or ± indomethacin (10 ppm) for 1 wk. (*A*) Representative pictures of TUNEL staining in the colon of control and sulindac-treated $BID^{+/+}$ and $BID^{-/-}$ $APC^{Min/+}$ mice, with arrows indicating example TUNEL-positive cells. (*B*) Representative pictures of active caspase 3 staining in the colon of control and sulindac- or indomethacin-treated $BID^{+/+}$ and $BID^{-/-}$ $APC^{Min/+}$ mice, with arrows indicating example active caspase 3-positive cells. (*C*) Analysis of caspase 8 activation by immunostaining. (*Upper*) Representative pictures of active caspase 8 staining in the small intestine and colon of control and sulindac- or indomethacin-treated $BID^{+/+}$ and $BID^{-/-}$ $APC^{Min/+}$ mice, with arrows indicating example active caspase 8-positive cells. (*Lower*) Mean + SD of active caspase 8 signals in the small intestinal and colonic crypts of the control (CT), sulindac (SUL)-, or indomethacin (INDO)-treated mice. (*D*) Representative pictures of TUNEL and Lgr5 (GFP) double staining in the colon of control and sulindac- or indomethacin-treated *Lgr5-EGFP*–marked $BID^{+/+}$ and $BID^{-/-}/APC^{Min/+}$ mice. (*E*) Analysis of intestinal stem cell apoptosis by *OLFM4* RNA in situ hybridization and TUNEL staining. (*Upper*) Representative staining in the small intestine of control and sulindac- or indomethacin-treated $BID^{+/+}$ and $BID^{-/-}$ $APC^{Min/+}$ mice, with arrows indicating TUNEL (green) and *OLFM4* (red) double positive cells. (*Lower*) Means + SD of *OLFM4*/TUNEL double-positive cells in the small intestinal crypts. (*F*) Plasma PGEM levels ($n = 3$ per group) were measured. $^{*}P < 0.05$; $^{**}P < 0.01$ ($n = 3$ per group). (Scale bars: 25 μm.)

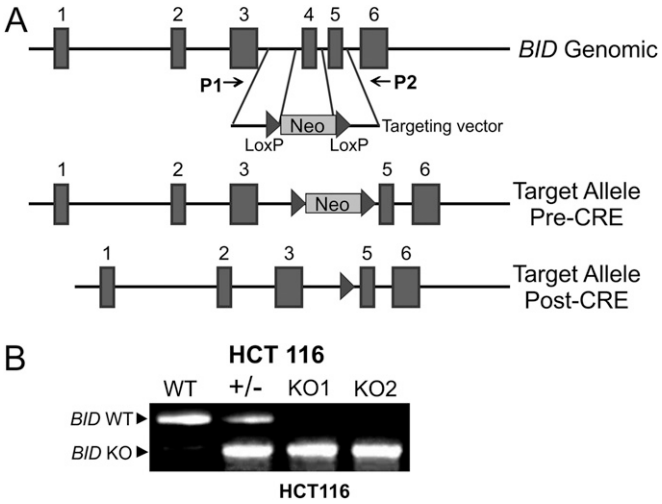

Fig. S3. Knockout of *BID* in HCT116 colon cancer cells. (*A*) Schematic representation of *BID* genomic locus and the targeting constructs for knocking out *BID*. P1 and P2 are PCR primers for identifying knockout clones. (*B*) Identification of *BID* knockout (KO) HCT116 cells by PCR analysis of genomic DNA. WT and KO alleles are indicated. KO1 and KO2 are two independent knockout cell lines.

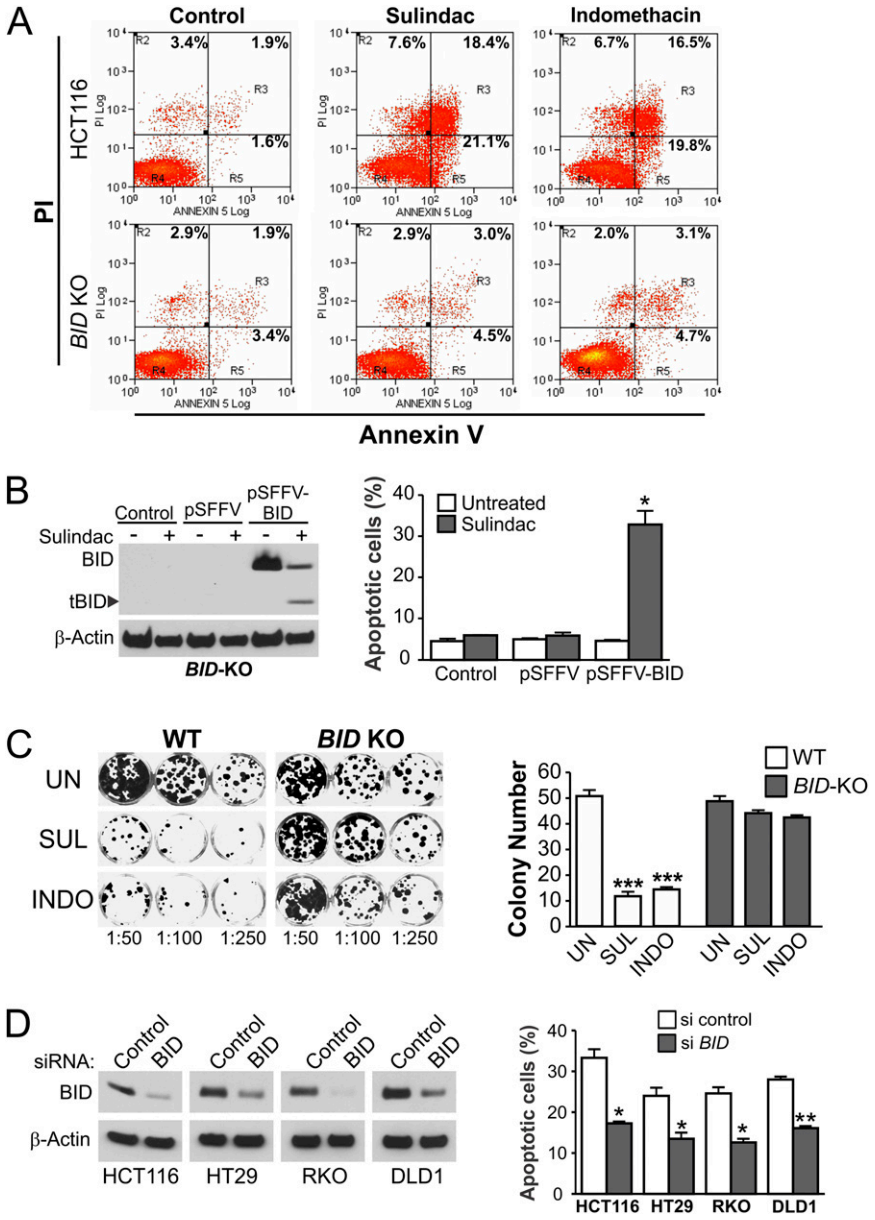

Fig. S4. NSAID-induced and BID-dependent apoptosis in colon cancer cells. (*A*) WT and *BID* KO HCT116 cells were treated with 120 μM sulindac sulfide or 500 μM indomethacin. Apoptosis in cells treated for 48 h was analyzed by annexin V/propidium iodide (PI) staining followed by flow cytometry. (*B*) *BID* KO cells were transfected with pSFFV-BID or empty pSFFV vector, and then treated with 120 μM sulindac sulfide for 24 h. (*Left*) Analysis of BID expression by Western blotting. (*Right*) Analysis of apoptosis by counting condensed and fragmented nuclei after nuclear staining with Hoechst 33258. (*C*) Colony formation assay was performed by seeding an equal number of WT and *BID* KO HCT116 cells treated with 120 μM sulindac sulfide (SUL) or 500 μM indomethacin (INDO) for 24 h at the indicated dilutions. Attached cells were stained with crystal violet 14 d later. (*Left*) Representative colony pictures after crystal violet staining. (*Right*) Quantification of colony numbers. (*D*) Indicated CRC cells were transfected with control or *BID* siRNA, and then treated with sulindac sulfide (HCT116, 120 μM; DLD1, 180 μM; HT29 and RKO, 200 μM) for 24 (HCT116, DLD1, and RKO) or 48 h (HT29). (*Left*) Western blot of BID in the transfected cells. (*Right*) Apoptosis was analyzed as in *B*. Results in *B*–*D* were expressed as means + SD of three independent experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

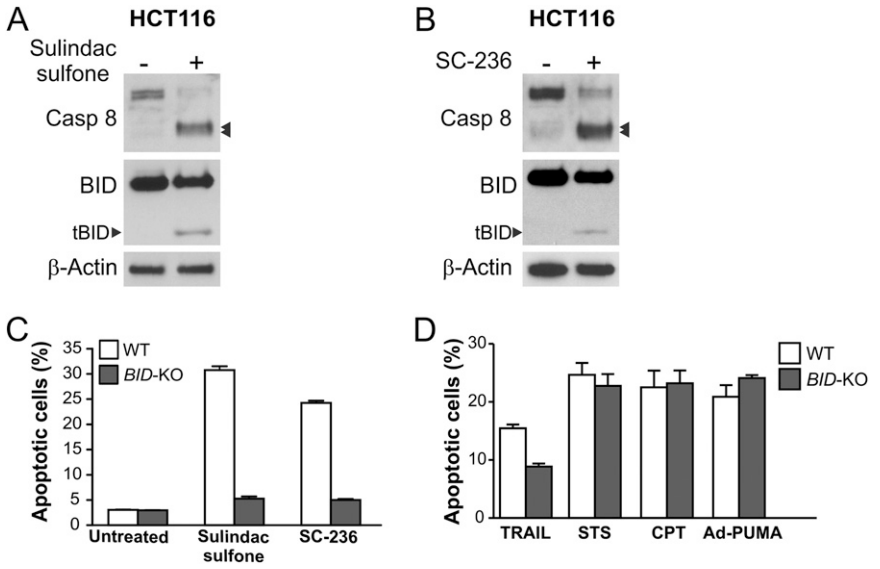

Fig. S5. BID is specifically required for NSAID-induced apoptosis. (*A*) Western blot of the indicated proteins in HCT116 cells treated with 400 μM sulindac sulfone for 24 h. (*B*) Western blot of the indicated proteins in HCT116 cells treated with 100 μM of the COX-2–specfic inhibitor SC-236 for 24 h. (*C*) Apoptosis in cells treated as in *A* and *B* was analyzed for apoptosis by counting condensed and fragmented nuclei after nuclear staining with Hoechst 33258. (*D*) WT and *BID* KO HCT116 cells were treated for 24 h with TRAIL (20 nM), STS (100 nM), CPT (400 nM) or an adenovirus-expressing PUMA (Ad-PUMA). Apoptosis was analyzed as in *C*.

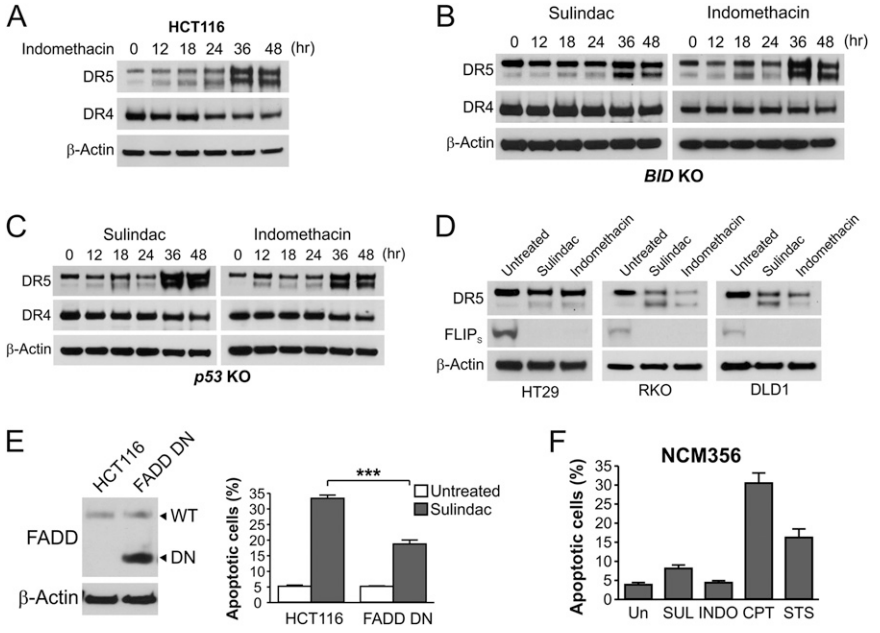

Fig. S6. Activation of the death receptor pathway by NSAIDs. (*A*) Western blot of DR5 and DR4 at the indicated time points in HCT116 cells treated with 500 μM indomethacin. (*B*) Western blot of DR5 and DR4 at the indicated time points in *BID* KO HCT116 cells treated with 120 μM sulindac sulfide or 500 μM indomethacin. (*C*) Western blot of DR5 and DR4 at the indicated time points in *p53* knockout (*p53* KO) HCT116 cells treated as in *B*. (*D*) Western blot of DR5 and $FLIP_S$ in HT29, RKO, and DLD1 cells treated with sulindac sulfide (DLD1, 180 μM; HT29 and RKO, 200 μM) or 500 μM indomethacin for 36 h. (*E*) Parental and stable HCT116 cells expressing dominant negative (DN) FADD were treated with sulindac sulfide as in *B*. (*Left*) Western blot analysis of indicated proteins at 24 h after treatment. (*Right*) Apoptosis at 24 h after treatment was analyzed by counting condensed and fragmented nuclei after nuclear staining with Hoechst 33258. ***$P < 0.001$. (*F*) NCM356 normal colonic epithelial cells were treated with sulindac sulfide (SUL; 120 μM), indomethacin (Indo; 500 μM), CPT (400 nM), or STS (100 nM) for 48 h. Apoptosis was measured as in *E*.

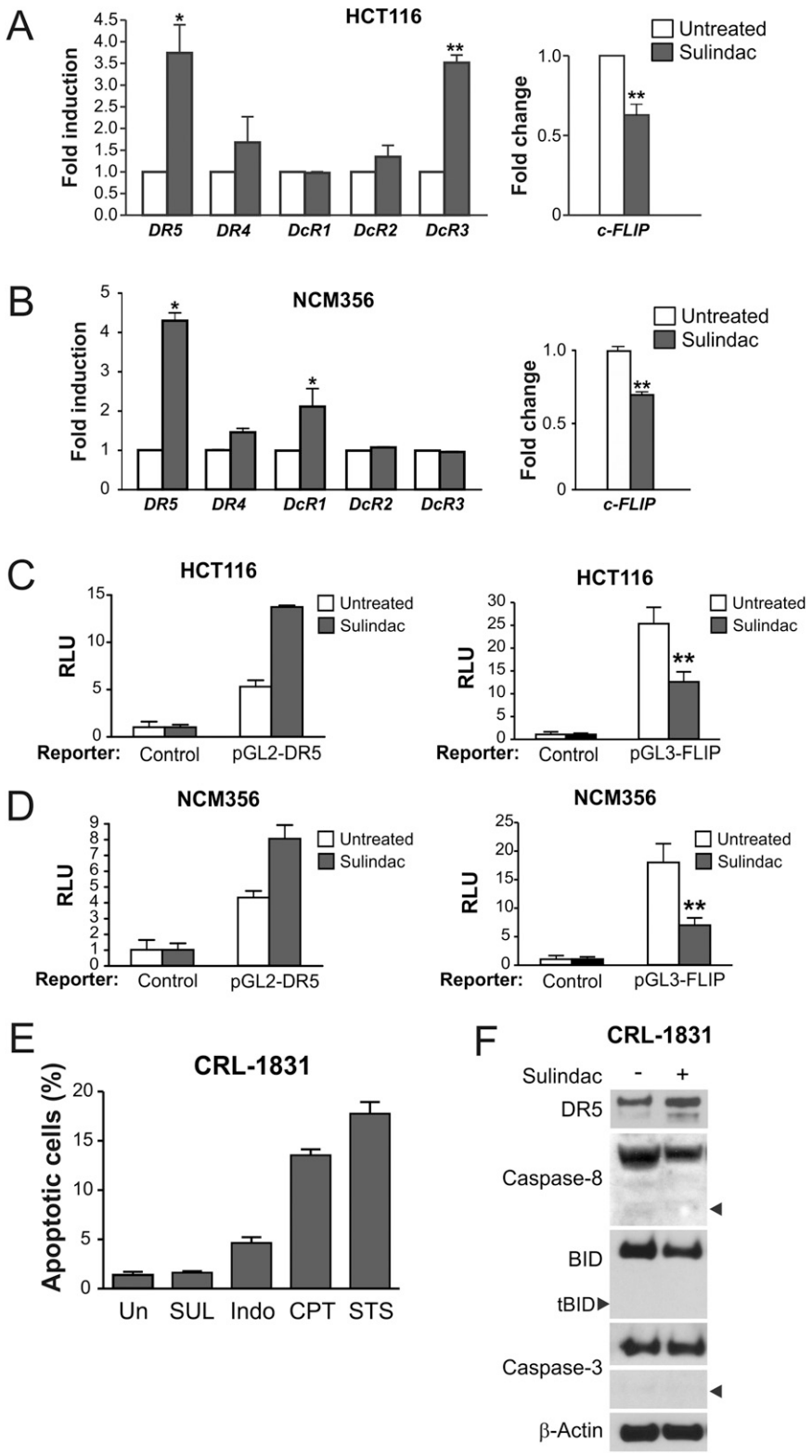

Fig. S7. Activation of death receptor signaling by NSAIDs in normal and cancer cells. (*A*) mRNA levels of the indicated genes were measured by real-time RT-PCR in HCT116 cells treated with 120 μM sulindac sulfide for 24 h. (*B*) mRNA levels of the indicated genes were measured by real-time RT-PCR in NCM356 cells treated as in *A*. (*C*) *DR5* and *c-FLIP* promoter activities in HCT116 cells transfected with the indicated plasmids and treated with 120 μM sulindac sulfide for 24 h. Firefly luciferase levels were normalized to β-galactosidase and expressed as relative luciferase units (RLU). (*D*) *DR5* and *c-FLIP* promoter activities in NCM356 cells transfected and treated as in *C*. (*E*) CRL-1831 normal colonic epithelial cells were treated with sulindac sulfide (SUL; 120 μM), indomethacin (Indo; 500 μM), CPT (400 nM), or STS (100 nM) for 48 h. Apoptosis was measured by counting condensed and fragmented nuclei after nuclear staining. (*F*) Western blot of indicated proteins in CRL-1831 cells treated with 120 μM sulindac sulfide for 24 h. Results in *A*–*D* were expressed as means + SD of three independent experiments. $*P < 0.05$; $**P < 0.01$.

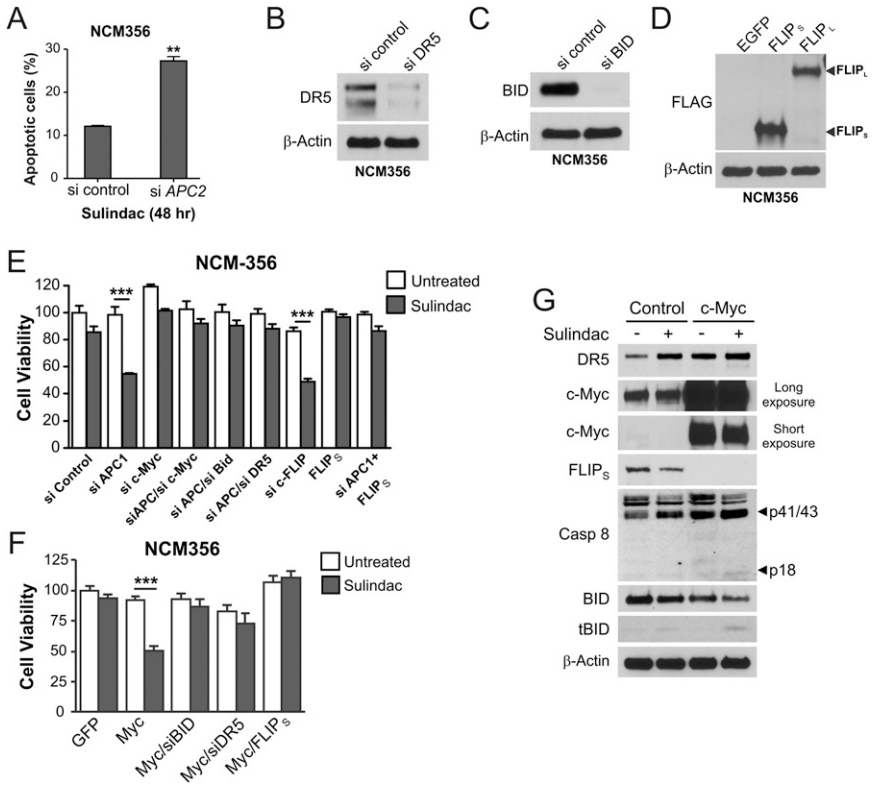

Fig. S8. APC depletion or c-Myc induction triggers BID activation and BID-dependent apoptosis in NSAID-treated NCM356 cells. (*A*) NCM356 cells were transfected with an independent *APC* siRNA (si *APC2*), and then treated with 120 μM sulindac sulfide for 48 h. Apoptosis was analyzed by counting condensed and fragmented nuclei after nuclear staining with Hoechst 33258. (*B* and *C*) NCM356 cells were transfected with *DR5* or *BID* siRNA for 48 h and then analyzed for their expression by Western blotting. (*D*) NCM356 cells were transfected with Flag-tagged c-FLIP$_L$ or c-FLIP$_s$ for 24 h, and then analyzed for c-FLIP expression by Western blotting. (*E* and *F*) NCM356 cells were transfected with indicated siRNA or siRNA/plasmid combinations, and then treated with 120 μM sulindac sulfide for 24 h. Cell viability was measured by using CellTiter-Glo assay. (*G*) Western blotting of the indicated proteins in NCM356 cells transfected with control or c-Myc expression vector, and then treated with 120 μM sulindac sulfide for 36 h. Results in *A*, *E*, and *F* were expressed as means + SD of three independent experiments. $^{**}P < 0.01$; $^{***}P < 0.001$.

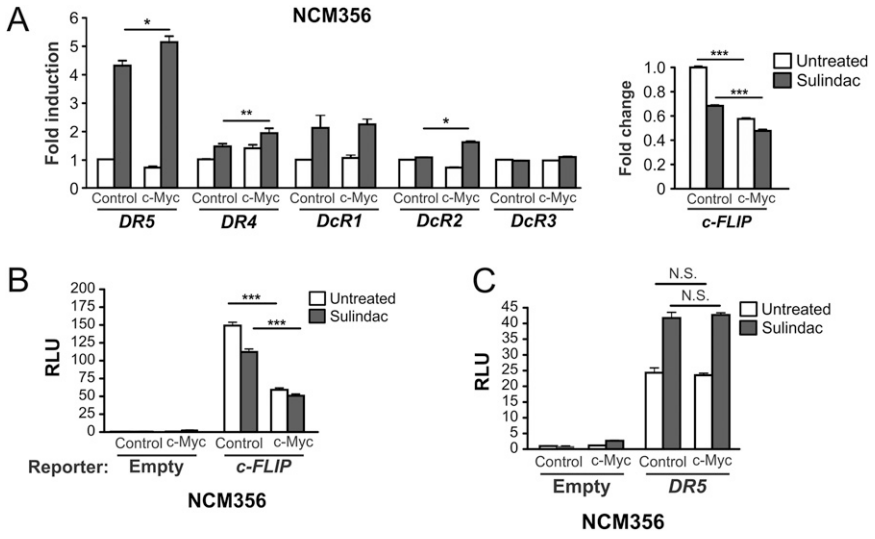

Fig. S9. The effects of c-Myc on the death receptor pathway regulators in NCM356 cells. (*A*) NCM356 cells were transfected with control or c-Myc expression vector, and then treated with 120 μM sulindac sulfide for 24 h. Indicated death receptor pathway regulators were analyzed by real-time RT-PCR. (*B*) *c-FLIP* promoter activities in NCM356 cells transfected and treated as in *A*. Firefly luciferase levels were normalized to β-galactosidase and expressed as relative luciferase units (RLU). (*C*) *DR5* promoter activity in NCM356 cells transfected and treated as in *A*. Results were expressed as means + SD of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; N.S., $P > 0.05$.