# Chemoprevention of colorectal cancer by targeting APC-deficient cells for apoptosis

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Cancer chemoprevention uses natural, synthetic, or biological substances to reverse, suppress, or prevent either the initial phase of carcinogenesis or the progression of neoplastic cells to cancer[1]. It holds promise for overcoming problems associated with the treatment of late-stage cancers. However, the broad application of chemoprevention is compromised currently by limited effectiveness and potential toxicity. In an effort to overcome these challenges, we developed a new chemoprevention approach that specifically targets premalignant tumor cells for apoptosis. This procedure is based on our finding that a deficiency in the *adenomatous polyposis coli* (*APC*) gene and subsequent activation of β-catenin lead to the repression of cellular caspase-8 inhibitor cFLIP expression through activation of c-Myc and that 9-cis-retinyl acetate (RAc) independently upregulates tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptors and suppresses decoy receptors. Thus, the combination of TRAIL and RAc induces apoptosis in APC-deficient premalignant cells without affecting normal cells *in vitro*. In addition, we show that short-term and non-continuous TRAIL and RAc treatment induces apoptosis specifically in intestinal polyps, strongly inhibits tumor growth, and prolongs survival in multiple intestinal neoplasms C57BL/6J-*Apc^Min*/J (*Apc^Min*) mice. With our approach, we further demonstrate that TRAIL and RAc induce significant cell death in human colon polyps, providing a potentially selective approach for colorectal cancer chemoprevention by targeting APC-deficient cells for apoptosis.

TRAIL, or Apo2L, is a membrane-bound TNF family ligand[2,3]. While TRAIL induces apoptosis in cancer cells, it does not harm normal cells[4,5]. To test the response of premalignant tumor cells to TRAIL, we isolated primary adenoma cells from intestinal polyps (benign adenomas) of *Apc^Min* mice[6-8]. We also isolated intestinal epithelial cells from wild-type mice as controls (Supplementary Fig. 1a). TRAIL treatment did not induce any apoptosis in the isolated epithelial cells (Fig. 1a and Supplementary Fig. 1b). Previous

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reports indicate that certain retinoids synergize TRAIL-induced apoptosis in some cancer cell lines by inducing either TRAIL or its death receptors [9-11]. We explored the effect of RAc on TRAIL sensitivity in isolated primary adenoma and normal epithelial cells. Although RAc alone had no effect on the survival of either normal cells from control mice or adenoma cells, it specifically sensitized the adenoma cells to TRAIL-induced cell death (Fig. 1a and Supplementary Fig. 1b). These results show that RAc and TRAIL synergistically induce apoptosis in intestinal adenoma cells derived from $Apc^{Min}$ mice.

The major difference between adenoma cells and normal cells is the loss of the wild-type *APC* allele in adenoma cells[12]. To test the role of APC in cell sensitivity to TRAIL and RAc, we knocked down APC expression in an immortalized normal human colon epithelial cell line, NCM356, using *APC* small interfering RNA (APC-siRNA) (Fig. 1b). We observed that knockdown of APC and treatment with TRAIL and RAc resulted in caspase 8 cleavage and an increase in caspase 3/7 activity (Fig. 1c and Supplementary Fig. 1c). Furthermore, caspase 8 inhibitor Z-IETD blocked caspase 3/7 activity (Supplementary Fig. 1c), suggesting that caspase 8 processing resulted in its activation. Similar results were obtained using an immortalized normal human bronchial epithelial cell line (BEAS-2B-BW1799 or BW1799) (Supplementary Fig. 2). These results demonstrate that APC deficiency is the key factor in sensitizing normal cells to apoptosis induced by the combination of TRAIL and RAc.

Inactivation of *APC* leads to stabilization and nuclear translocation of β-catenin and transcriptional activation of target genes, such as *c-Myc* and *cyclin D1*[13]. To investigate the signaling events in the APC pathway required for sensitizing cells to TRAIL and RAc, we transfected either stabilized β-catenin[14] or full-length *c-Myc* into NCM356 cells (Supplementary Fig. 3a and b). Expression of either β-catenin mutant or c-Myc led to TRAIL- and RAc-mediated caspase 8 cleavage and activation (Fig. 2a and Supplementary Fig. 3c). Transfection of cyclin D1 had no effect on TRAIL- and RAc-induced apoptosis in NCM356 and other cells (data not shown). We then determined the requirement of c-Myc for sensitizing cells to TRAIL and RAc. Knockdown of APC led to an increase in c-Myc expression, as expected. Co-transfection of c-Myc-siRNA blocked APC deficiency-mediated c-Myc induction and caspase 8 cleavage/activation in response to TRAIL and RAc (Fig. 2b and Supplementary Fig. 3c). We further confirmed these observations using independent siRNAs (Supplementary Fig. 4). These results indicate that the effect of APC on cell response to TRAIL and RAc is largely dependent on APC/β-catenin-mediated activation of c-Myc.

Other studies have shown that c-Myc directly suppresses expression of cFLIP and that this activity correlates with TRAIL sensitivity[15]. NCM356 cells expressed readily detectable cFLIP and transfection of c-Myc resulted in a significant decrease in cFLIP expression and caspase 8 cleavage (Fig. 2c). While direct suppression of cFLIP expression by cFLIP-siRNA led to caspase 8 cleavage and activation in NCM356 cells treated with TRAIL and RAc, rescue of cFLIP expression by transfection of a cFLIP expression plasmid prevented c-Myc-induced caspase 8 processing and activation in response to TRAIL and RAc (Fig. 2c and Supplementary Fig. 3c). Similar results were obtained in BW1799 cells (Supplementary Fig. 5). In addition to suppressing cFLIP expression, c-Myc could also act on mitochondria to facilitate TRAIL-induced apoptosis[16]. To test this possibility, we performed rescue experiments using Bcl-2 and Bcl-XL. Overexpression of either Bcl-2 or Bcl-XL did not inhibit c-Myc-mediated caspase 8 processing and activation in response to TRAIL and RAc (Fig. 2c and Supplementary Fig. 3c). These results indicate that APC deficiency sensitizes normal cells to TRAIL and RAc by suppressing cFLIP expression through the activation of β-catenin and c-Myc.

Previous reports have shown that certain retinoids sensitize some cancer cells to TRAIL-induced apoptosis by upregulating either TRAIL or TRAIL receptors DR4 and DR5 [9,10]. To test the effect of RAc on normal cells in our setting, we examined the expression of TRAIL receptors in NCM356, BW1779, and another immortalized normal human colon epithelial cell line CRL1831 cells after RAc treatment. Although RAc did not induce TRAIL expression (data not shown), it induced DR4 or DR5 expression in these cells (Fig. 2d and Supplementary Fig. 6a). Interestingly, RAc strongly suppressed DcR1 expression in these cells (Fig. 2d and Supplementary Fig. 6a). In addition, the DcR2 expression was inhibited by RAc in NCM356 cells (Fig. 2d). We further showed that RAc caused similar changes in mRNA levels in NCM356 cells (Supplementary Fig. 6b), suggesting that RAc modulates TRAIL receptor expression at the levels of mRNA.

Overexpression of DR5 sensitized APC-knockdown cells to TRAIL-induced caspase 8 cleavage and activation (Fig. 2e and Supplementary Fig. 6c). Pretreatment of APC-knockdown cells with a DcR1-neutralizing antibody to block TRAIL-DcR1 interaction also partially induced caspase 8 cleavage and activation after TRAIL treatment, and this effect was further enhanced in DR5-transfected cells (Fig. 2e and Supplementary Fig. 6c). Similar results were obtained in BW1799 cells (Supplementary Fig. 6d and e). These data suggest that both induction of DR5 and suppression of DcR1 contribute to the effect of RAc in sensitizing APC-deficient cells to apoptosis.

We next tested the effect of TRAIL and RAc in $Apc^{Min}$ mice *in vivo*. We first treated the 3-month-old $Apc^{Min}$ mice with TRAIL and low-dose RAc for 15 treatment cycles over six weeks. We observed that TRAIL and RAc significantly inhibited the growth of intestinal polyps as compared to controls (Fig. 3a). Histological analysis of tumors and adjacent normal tissues in treated mice showed that many cells in tumors, but not in normal tissues, contain condensed nuclei, suggesting that cells in the tumors are dying (Fig. 3b). This observation was confirmed by terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) analysis (Fig. 3b). When the total numbers and sizes of the polyps were compared 1 month after the six week treatment period, the treated mice displayed an average 4- to 5-fold decrease in the total number of polyps relative to the mice treated with PBS control (Fig. 3c). The reduction in large polyps ( 2 mm) was much more pronounced (to about 1/30 of control) (Fig. 3c). These data demonstrate that the combination of TRAIL and RAc induces apoptosis specifically in *Apc*-defective polyps and effectively suppresses the tumor growth.

Biochemically, accumulation of soluble β-catenin and induction of c-Myc expression were seen in the polyps of $Apc^{Min}$ mice but not in normal tissue (Supplementary Fig. 7a). cFLIP protein was readily detectable in normal tissue, while it was significantly reduced in polyps (Supplementary Fig. 7b). Consistent with our *in vitro* observations, RAc induced DR5 expression and repressed DcR1 in both the polyps in $Apc^{Min}$ mice.

Since TRAIL and RAc together target APC-deficient cells for apoptosis, this combination has the potential to achieve chemoprevention in short-term therapy. To test this, we applied two short consecutive TRAIL and RAc treatment cycles to $Apc^{Min}$ within a week and analyzed the intestinal tumors 2 weeks after treatment. After only two treatments, we observed a 69% reduction in the number of polyps when 3 mg/kg of TRAIL and 68 μg/kg of RAc were injected into $Apc^{Min}$ mice (Fig. 3d). The tumor numbers were further reduced to 10% of the control when 10-fold higher doses of TRAIL and RAc were used (Fig. 3d). These data indicate that short-term treatment with TRAIL and RAc is capable for inhibiting growth of premalignant cells in $Apc^{Min}$ mice. To test the effect of TRAIL and RAc treatment on the survival of $Apc^{Min}$ mice, we applied 5 non-consecutive TRAIL and RAc treatments to $Apc^{Min}$ mice within 4 months (average 1 treatment every 3 weeks). All mice in

the control group treated with PBS died within 7 months; however, five of the seven treated mice survived beyond 8 months (P 0.001) (Fig. 3e) demonstrating that non-continuous TRAIL and RAc treatment yields a long-term survival benefit for $Apc^{Min}$ mice. While it is likely that the two early deaths in the treatment group resulted from a late start in treatment, it cannot be ruled out that intrinsic resistance, heterogeneity, genetic or epi-genetic changes of premalignant cells can influence the effect of TRAIL treatment. Further study will provide a more in-depth assessment.

As a first step to demonstrate the efficacy and relevance of TRAIL and RAc treatment in humans, we treated biopsy samples of both normal and tumor regions from consenting familial adenomatous polyposis (FAP) patients under organ culture conditions as reported [17,18]. A cross section of normal colon tissue showed a round and defined structure of individual villus consisting of a monolayer of colon epithelial cells and membrane β-catenin staining pattern, while dysplastic colon polyps showed much larger villi, bigger nuclei, an irregular structure consisting of multiple epithelial layers, and enhanced nuclear β-catenin staining (Fig. 4a). Treatment of normal tissue slices with TRAIL and RAc did not induce significant cell death, while the same treatment in the polyp samples resulted in significant cell death, as detected by TUNEL assay (Fig. 4a). Quantification of TUNEL-positive cells showed that TRAIL and RAc killed an average of 57% of the cells in the human colon polyps (Fig. 4b). These results verify that TRAIL and RAc are effective against human polyps from FAP patients *in vitro* and that our approach holds promise for the chemoprevention of colon cancer in humans.

A major concern in chemoprevention is potential toxicity associated with treatment. We have shown that TRAIL and RAc target the APC-β-catenin-c-Myc signaling pathway for apoptosis. Since activation of β-catenin is involved in stem cell self-renewal and maintenance in adult tissues[19], TRAIL and RAc could possibly interact in a detrimental way with stem cells. In order to investigate this, we first tested the effect of TRAIL and RAc on intestinal stem cells (ISCs) in $Apc^{Min}$ mice. Using a putative ISC marker leucine-rich-repeat-containing G-protein-coupled receptor (Lgr5)[20], we noted that most of the Lgr5 positive cells in the normal sections were located at the bottom of the crypt as reported[20] (Fig. 4c). Staining of active caspase 3 revealed extensive staining in tumor sections similar to the findings shown in Figure 3b (Fig. 4c). More importantly, no staining was detected in serial sections from the normal region, including Lgr5+ cells in the crypt regions where the ISC resides (Fig. 4c). We also studied the effect of long-term TRAIL and RAc treatment on tissue-resident stem cells isolated from pararenal adipose tissue (mASCs) of $Apc^{Min}$ mice[21] (Supplementary Fig. 8). We found no detectable effect on morphology (data not shown), neural differentiation (data not shown), proliferation and apoptosis, senescence, osteogenesis, and adipogenesis both *in vitro* and *in vivo* (Supplementary Fig 9 and 10). Taken together, these results suggest that TRAIL and RAc exert no negative effect on stem cells (ISCs and mASCs) in mice.

In summary, we discovered a synergistic interaction among TRAIL, RAc, and tumor suppressor APC, which results in the specific induction of apoptosis in APC-deficient cells by TRAIL and RAc (Supplementary Fig. 11). Induction of apoptosis represents a most potent cellular mechanism against cancer, and selectively eliminating premalignant tumor cells by TRAIL and RAc is an effective method of chemoprevention. More importantly, we demonstrate that the chemopreventive effect of TRAIL and RAc can be achieved by short-term intermittent and non-continuous treatment cycles. Thereby, the potential side effects and costs often associated with long-term treatment could be minimized and controlled.

## Methods Summary

Recombinant soluble human TRAIL was prepared according to a published method[22]. For TRAIL and RAc treatment, the cells were first treated with RAc (6.8 ng/ml) for 48 h, and then TRAIL (50ng/ml or 500ng/ml) was added. Cell viability was determined using Annexin V-FITC Apoptosis Detection Kit (Sigma). Caspase 3/7 activity was measured using Apo-ONE Homogeneous Caspase 3/7 assay kit (Promega). Transfection and Western blotting were performed as described previously[23]. The apoptotic cells in tumor samples were detected by TUNEL assay using an apoptosis detection kit (R&D Systems) or caspase 3 staining using an anti-cleaved caspase 3 antibody (Cell Signaling). $Apc^{Min}$ mice were purchased from the Jackson Laboratory. For treatment, $Apc^{Min}$ mice at 3 months of age were first given RAc via i.p. injection at 68 μg/kg. After 48 h, TRAIL was injected i.p. at 3 mg/kg. After 24 h, RAc was injected again, followed by TRAIL injection. The procedure was repeated for 6 weeks resulting in a total of 15 treatment cycles. Whole intestines were collected immediately after euthanasia and opened longitudinally so that polyps could be counted and measured. Tissues were processed by either formalin fixation or by being frozen in liquid nitrogen for tissue protein extraction, H&E staining, immunohistochemical analysis, and TUNEL assay. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of The University of Texas M. D. Anderson Cancer Center. Fresh human tissue samples were obtained from consenting FAP patients according to an Institutional Review Board-approved protocol.

## Methods

### Cell lines and transfection

Primary mouse intestinal epithelial cells and adenoma cells were isolated from either normal C57/B6 mice or $Apc^{Min}$ mice as reported[24]. Briefly, the small intestine or polyps were cut into 2- to 3-mm segments. The tissues were transferred to a 15-ml tube, washed at least 10 times in 10 ml of fresh Hank's buffered salt solution (HBSS) with vigorous shaking, and diced into <1-mm$^3$ pieces using a sharp scalpel blade. The tissue pieces were transferred to a 15-ml tube with 10 ml Dulbecco's modified Eagle's medium (DMEM) containing 100 U/ml penicillin and 100 μg/ml streptomycin. The mixtures were allowed to settle under gravity for 1 min and all but a small amount at the bottom was carefully removed. This procedure was repeated 5 times. The mixtures were then washed with DMEM 3 times and the pellet was resuspended in the epithelial cell medium containing equal volumes of DMEM and Ham's F-12 medium with the following additives: 5 μg/ml insulin, 10 ng/ml epidermal growth factor, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.2% D-glucose, and 2% fetal bovine serum (FBS). CRL1831 cells were obtained from ATCC. NCM356 cells were obtained from INCELL (San Antonio, TX) and grown in A52 medium supplemented with 30 μg/ml bovine pituitary extract, 8 μg/ml vitamin C, 2 mM glutamine and 1 nM dexamethasone. A52 was custom made without folic acid, which was added at concentrations of 25 nM, 50 nM, 75 nM and 150 nM before use. BW1799 cells were kindly provided by Dr. Reuben Lotan of The University of Texas M. D. Anderson Cancer Center and maintained in keratinocyte-SFM medium (Gibco).

### Antibodies and other materials

Anti-full length and anti-cleaved caspase 8, anti-cleaved caspase 3, anti-cFLIP, anti-DR5, anti-Bcl-2, and anti-Bcl-XL antibodies were purchased from Cell Signaling Technology. Anti-c-Myc and anti-cytokeratin-20 (CK-20) antibodies were purchased from Santa Cruz Biotechnology Inc. Anti-APC was obtained from Calbiochem. Anti-β-actin and anti-α-tubulin were purchased from Sigma. Anti-DcR1 and anti-DcR2 antibodies were purchased

from Imgenex. Anti-DR4 antibody was obtained from Upstate. Anti-β-catenin antibody was purchased from BD Transduction Laboratories. Fluorescein isothiocyanate-conjugated anti-mouse CD14 and CD31 and phycoerythrin-conjugated anti-mouse CD44 and CD90 antibodies were purchased from BD Pharmingen. Anti-Lgr5 antibody was obtained from Abcam. Recombinant soluble TRAIL was prepared according to published results[22]. RAc was purchased from Sigma.

### Plasmids and vector-based siRNA construction

The c-Myc, β-catenin, Bcl-2, and Bcl-XL expression plasmids were described previously[14,23,25]. DR5 expression plasmid was kindly provided by Dr. Wafik El-Deiry of the University of Pennsylvania School of Medicine. The full-length cFLIP cDNA was generated by reverse-transcription polymerase chain reaction of total RNA from HeLa cells. All siRNAs except APC-siRNA(2) and c-Myc-siRNA(2) were generated in pSUPER as described elsewhere[23]. The target sequence of human APC is 5′-ggaagtattgaagatgaag-3′, of human cFLIP 5′-agaggtaagctgtctgtcg-3′, and of human c-Myc 5′-ttcaagaga-3′. The APC-siRNA(2) and c-Myc-siRNA(2) were purchased from Open Biosystems with target sequences of 5′-gctgtgaaattcacagtaata-3′ and 5′-ccgagaacagttgaaacacaaa-3′, respectively.

### Quantitative PCR

Total RNA was extracted using Trizol reagent according to the manufacturer's instructions (Invitrogen). The cDNA was prepared using SuperScript cDNA Synthesis Kit (Invitrogen). Quantitative PCR was performed using the SYBR Green Quantitative PCR kit from Thermo Scientific in a Bio-Rad C1000TM Thermal Cycler following the manufacturer's protocol. Amplification was carried out in a total volume of 20 μL for 40 cycles of 15 seconds at 95°C, 20 seconds at 60°C, and 30 seconds at 72°C. Samples were run in triplicate and their relative expression was determined by normalizing expression of each target GAPDH. These were then compared with the normalized expression in Control untreated sample to calculate a fold change value. Primer sequences were as follows: human GAPDH 5'-TGCACCACCAACTGCTTAGC-3' and 5'-GGCATGGACTGTGGTCATGAG-3'; human DR4 5'-TGTACGCCCTGGAGTGACAT-3' and 5'-CACCAACAGCAACGGAACAA-3'; human DR5 5'-CACTCACTGGAATGACCTCCTTT-3' and 5'-GTGCAGGGACTTAGCTCCACTT-3'; human DcR1 5'-CCCTAAAGTTCGTCGTCGTCAT-3' and 5'-GGGCAGTGGTGGCAGAGTA-3'; human DcR2 5'-ACAGAGGCGCAGCCTCAA-3' and 5'-ACGGGTTACAGGCTCCAGTATATT-3',

### *Ex vivo* organ cultures

Paired tumor and adjacent normal samples from consenting patients (male and female) undergoing colonoscopy at the MD Anderson Cancer Center Gastrointestinal Oncology and Digestive Diseases endoscopy and surgical unit were collected, transferred to research lab in cold media, and processed within 1 h in a sterile tissue culture hood. The culture condition is modified based on previous reports[17,18]. Briefly, the culture medium consists of a 1:1 mixture Dulbecco's modified Eagles's medium and Ham's F12 with 5% fetal calf serum (FCS), 10mM HEPES, 0.5% DMSO, 0.5ug/ml Hydrocortisone, 1% MEM vitamins solution, 5μg/ml insulin, 5μg/ml transferin, 5ng/ml selenium, 100 IU/ml penicillin, 100 μg/ml streptomycin, 15 μg/ml gentamicin and 15 μg/ml Ciproxin. The tissue is washed five times in culture media. The tissue is placed in a sterile glass dish where all necrotic and visible connective tissue is removed. The tissue is sliced gently into very thin pieces using a blade and placed in a 6-well tissue culture dish with one ml of growth media. The dish is placed in a 37°C, 5% $CO_2$ incubator for 24 h. It is inspected visually for contamination and the media is pulled off and replaced after every 2 days. After 1 day in culture, the tissue slices were subject to various treatment protocols.

### Isolation and culture of adipose tissue-derived stem cells

Subcutaneous adipose tissue was obtained from three mice (one wild-type and two $Apc^{Min}$) or $Apc^{Min}$ mice that had received 5 cycles of TRAIL and RAc treatment within 4 months following our institutional guidelines. Cells were isolated from adipose tissue as described previously [26,27]. Adipose tissue was minced and incubated for 90 min at 37°C on a shaker with Liberase Blendzyme 3 (Roche) at a concentration of 4 U/g fat tissue in phosphate-buffered saline (PBS). The digested tissue was sequentially filtered through 100-$\mu$m and 40-$\mu$m filters (Fisher Scientific) and centrifuged at 450 g for 5 min. The supernatant containing adipocytes and debris was discarded; the pelleted cells were washed twice with HBSS (Cellgro) and finally resuspended in stem cell growth medium (SCGM), which contained alpha-modification of Eagle's medium ($\alpha$MEM, Cellgro), 20% FBS (Atlanta Biologicals), 2 mM glutamine (Cellgro), and 100 U/ml penicillin with 100 $\mu$g/ml streptomycin (Cellgro). Cells adhering to plastic were designated mice adipose tissue-derived stem cells (mASCs) and grown in Nunclon culture flasks (Nunc) at 37°C in a humidified atmosphere containing 5% $CO_2$, followed by daily washes to remove red blood cells and non-attached cells. After the mASCs became confluent (passage 0), cells were digested and seeded at a density of 3,000 cells/cm$^2$ (passage 1).

### Assays of mASCs

For *in vitro* treatment assays, mASCs were cultured in the presence TRAIL (50ng/ml), or RAc (6.8 ng/ml), or both for two months (about 60 doublings). For effect of TRAIL and RAc treatment *in vivo*, mASCs were harvested from $Apc^{Min}$ mice that had received 5 cycles of treatments with TRAIL (3 mg/kg) and RAc (68 $\mu$g/kg) in 4 months. Cell proliferation was determined by counting live cells. Apoptosis was measured using Annexin V staining (BD Biosciences) using a FACSCalibur flow cytometer (Becton Dickinson) and Cell Quest software (Becton Dickinson). Cell senescence was determined by $\beta$-galactosidase activity at pH 6 using the Senescent Cells Staining Kit (Cell Signaling) according to the provided protocol. Osteogenic differentiation of mASCs was induced in osteogenic medium containing high-glucose DMEM supplemented with 10% FBS, 0.1 $\mu$M dexamethasone, 200 $\mu$M L-ascorbic acid, and 10 mM $\beta$-glycerol phosphate (Sigma). The medium was changed every 3 days for 2 weeks. In the control experiment, mASCs were cultured in control medium containing high-glucose DMEM plus 10% FBS for 2 weeks. To assess mineralization, calcium deposits in cultures were stained with Alizarin Red S (Sigma). The osteogenic efficiency was then determined using Osteogenesis Quantitation Kit (Chemicon) following the manufacture's instructions. The adipogenic ability of mASCs was induced in adipogenic medium for up to 2 weeks as described previously[26,27]. The adipogenic medium contained low-glucose DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin (Cellgro), 100 $\mu$M L-ascorbic acid, 1 $\mu$M dexamethasone, 0.5 mM 1-methyl-3-isobutylxanthine, 100 $\mu$M indomethacin, and 10 $\mu$g/ml insulin (Sigma). mASCs of the control group were cultured in low-glucose DMEM plus 10% FBS (control media). The medium was changed every 3 days. Adipogenesis of cells was assessed by incubating cells with Oil Red O solution (Sigma) to stain neutral lipids in the cytoplasm. The adipogenic efficiency of mASCs was then determined using Adipogenesis Quantitation Kit (Chemicon) according to the protocol provided by the manufacturer.

### Immunohistochemistry and Immunofluorescence staining

Tissues in paraffin block were sectioned at 4-$\mu$M in thickness. After deparaffinization, Antigen retrieval was performed using citrate buffer (Vector lab, Burlingame, CA) heated in a pressure cooker for 25 min and then cooled to room temperature. Blocking of endogenous peroxides was accomplished by incubating sections in 3% hydrogen peroxide (Sigma) for 5 min. Antibodies were incubated with sections overnight at 4°C. Immunostaining was

performed by using DAB (3,3'-diaminobenzidine) or AEC (3-Amino-9-ethylcarbazole) (Dako) according to the manufacturer's instructions. Sections were counterstained with hematoxylin for 1 min, rinsed in water and cover slipped permanently for light microscopy.

The mASCs cultured on glass cover slips were washed 3 times with PBS and fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature. Cells were then washed 3 times with PBS alone or PBS containing 0.3% Triton X-100 (Sigma) and blocked by treatment with 10% donkey serum for 30 min at room temperature. Cells were then incubated with primary antibodies fluorescein isothiocyanate-conjugated anti-mouse CD14 and CD31 or phycoerythrin-conjugated anti-mouse CD44 and CD90 in a humidified chamber for 1 h at 37°C. Thereafter, 4',6-diamidino-2-phenylindole (DAPI) was used to stain the cell nuclei. The images of cells were taken under a fluorescence microscope.

## Statistical analysis

The two-tailed Student's t-test or one-way analysis of variance (ANOVA) was used to compare differences between groups. Values with $P < 0.05$ were considered statistically significant. Survival analyses were performed using SPSS software (Version 16.0).

## Acknowledgments

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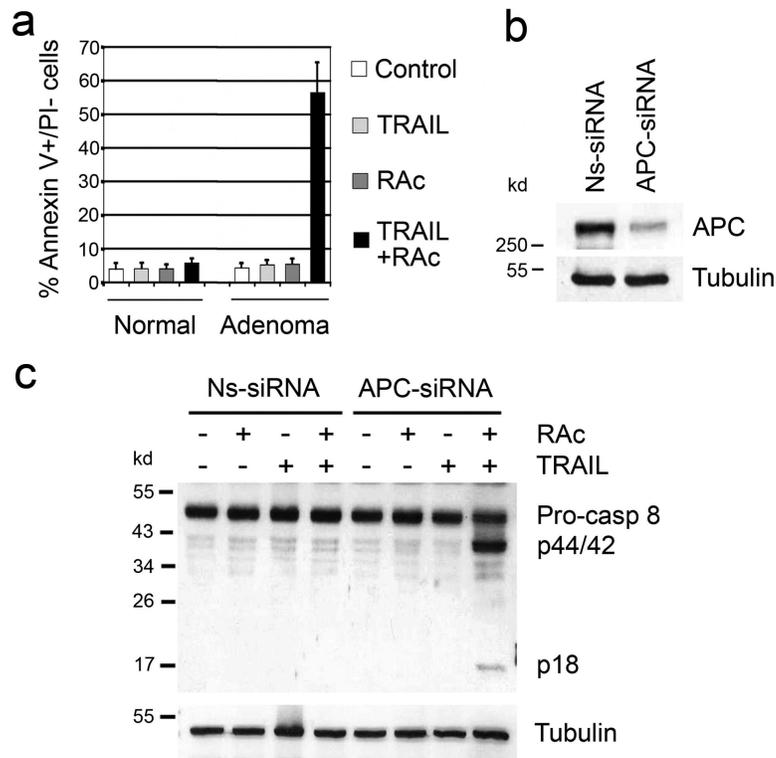
**Figure 1. TRAIL and RAc induce apoptosis in APC-deficient cells**
(a) Primary epithelial cells were isolated from normal mouse intestine and polyps from
$Apc^{Min}$ mice and treated with TRAIL (50 ng/ml for 24 h), RAc (6.8 ng/ml for 48 h), or both
(RAc for 48 h then TRAIL for 24 h). Cells were stained with annexin V-FITC and
propidium iodide. Early apoptotic cells (annexin V positive, PI negative) were counted. The
data represent results from three independent experiments. Average and standard deviation
are shown. (b) Immortalized normal human colon epithelial cells (NCM356) were
transfected with either nonspecific Ns-siRNA or APC-siRNA, and APC protein was
detected 48 h after transfection by Western blotting. (c) The transfected NCM356 cells were
treated and harvested, and both full-length pro-caspase 8 and cleaved forms (p44/42 and
p18) were detected by Western blotting.
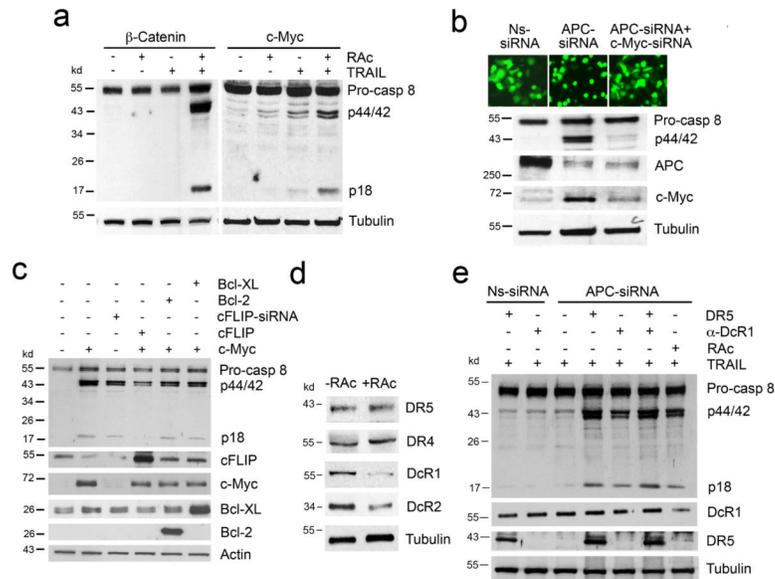
**Figure 2. Down regulation of cFLIP by APC/β-catenin-mediated activation of c-Myc and modulation of TRAIL receptor expression by RAc contribute to the activation of TRAIL signaling**

(a) NCM356 cells were transfected with either β-catenin or c-Myc and treated with TRAIL, RAc, or both. Cleavage of caspase 8 was detected by Western blotting. (b) NCM356 cells were transfected with Ns-siRNA, APC-siRNA (GFP tagged), or APC-siRNA and c-Myc-siRNA. The transfected cells were treated with RAc and TRAIL, and photographs were taken. Expression of APC, c-Myc, and caspase 8 was detected by Western blotting. (c) NCM356 cells were transfected with c-Myc, cFLIP-siRNA, c-Myc and cFLIP, c-Myc and Bcl-2, or c-Myc and Bcl-XL. The transfected cells were treated with RAc and TRAIL. Expression of c-Myc, cFLIP, Bcl-2, Bcl-XL, and caspase 8 was detected by Western blotting. (d) NCM356 cells were treated with RAc for 48 h, and expression of DR4, DR5, DcR1, and DcR2 was detected by Western blotting. (e) NCM356 cells were transfected with either APC-siRNA or APC-siRNA and DR5 or were pretreated with an anti-DcR1 antibody after APC-siRNA transfection. Nonspecific Ns-siRNA was used as control. Transfected cells were treated with TRAIL alone or with TRAIL and RAc, as indicated. Cleavage of caspase 8 was detected by Western blotting.
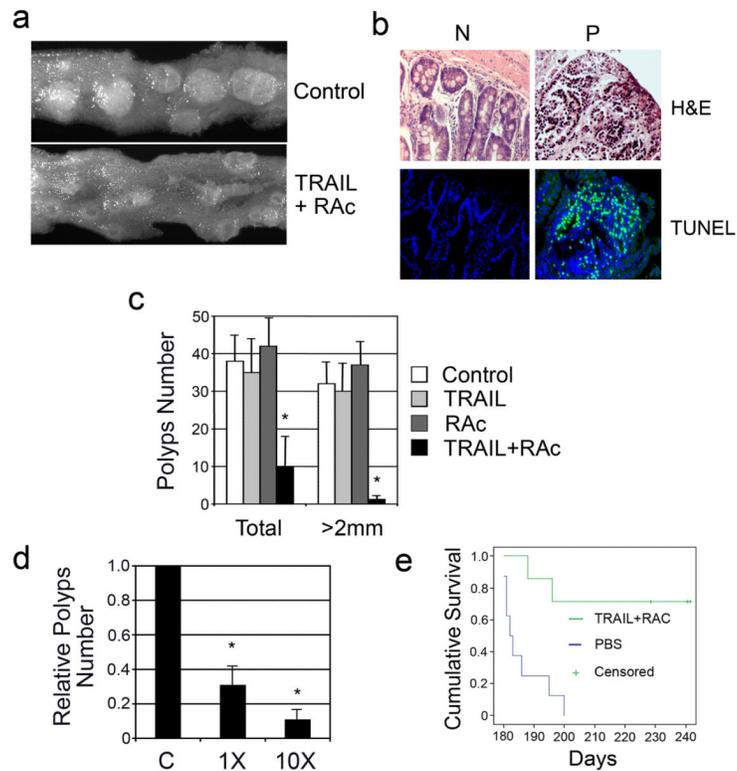
**Figure 3. Treatment with TRAIL and RAc induces cell death in the polyps, inhibits tumor growth, and promote survival in $Apc^{Min}$ mice**

(a) The $Apc^{Min}$ mice were treated with either control phosphate buffered saline (PBS) or TRAIL and RAc for 15 treatment cycles. Intestinal polyps were examined 1 month after the treatment and photographs were taken. (b) The polyp samples and adjacent sections were stained by H&E and TUNEL stain. Surrounding normal tissue was used as the control. Representative photographs are shown. (c) Intestinal polyps in the entire intestinal track were examined and counted. Each treatment group contained 5-7 mice. Average and standard deviations are shown. An asterisk indicates P<0.005. (d). The $Apc^{Min}$ mice were treated with either 1X or 10X doses of TRAIL and RAc for two cycles within a week. The intestinal polyps in the entire intestinal track were examined and counted. Each treatment group contained 5-7 mice. Average and standard deviation are shown. An asterisk indicates P<0.005. (e) Kaplan-Meier survival analysis of $Apc^{Min}$ mice. $Apc^{Min}$ mice at 4 month were treated with either TRAIL (3 mg/kg) and RAc (68 μg/kg) or control PBS every 3 weeks for 5 times. The endpoint was reached when mice were either moribund or at day 243. Mean survival time was 212.8 ± 5.9 in TRAIL and RAc group (7 mice) and 186 ± 2.6 in the PBS treated group (8 mice) with log-rank test p 0.001. All statistical analyses were performed using SPSS software (version 16.0).
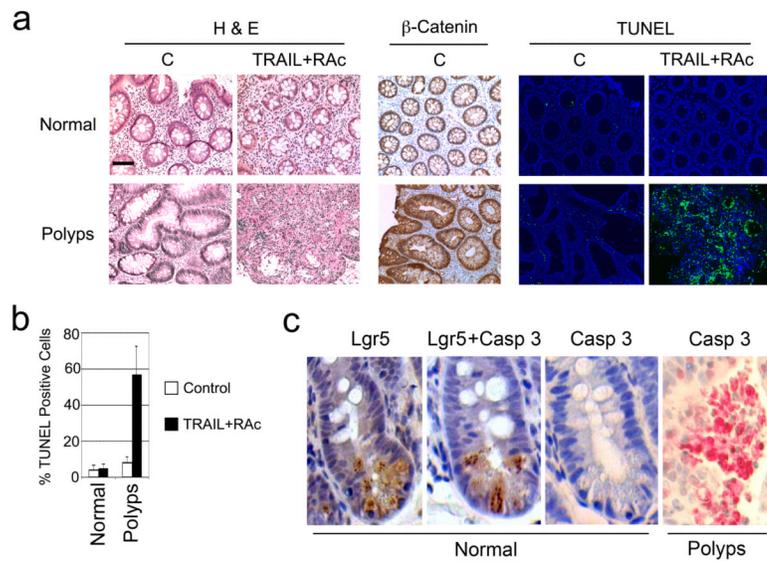
**Figure 4. Effect of TRAIL and RAc on human colon polyps and ISCs, and synthetic lethal interaction of TRAIL, RAc and APC**

(a) Tissue slices from both the normal region and colon polyps from FAP patients were treated with RAc (6.8 ng/ml) for 48 h and then with TRAIL (100 ng/ml) for an additional 24 h (TRAIL+RAc). The samples treated with vehicle were used as controls (C). H&E staining, β-catenin staining, and TUNEL assay (green) were performed. Nuclei were revealed by DAPI staining (blue). Scale bar equals 100 μm. Representative photographs are shown. (b) TUNEL-positive cells (green) were counted against the number of nuclei in multiple fields under a microscope. Average and standard deviations are shown. The data are derived from samples of 4 patients. (c) $Apc^{Min}$ mice were treated with 5 non-continuous cycles of TRAIL and RAc. Both the normal tissue and polyps of the intestine were serially sectioned and stained with either Lgr5 (brown), or activated caspase 3 (red), or both. Multiple serial sections were analyzed and representative photographs are shown.