Chemoprevention by nonsteroidal anti-inflammatory drugs eliminates oncogenic intestinal stem cells via SMAC-dependent apoptosis

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Nonsteroidal anti-inflammatory drugs (NSAIDs) such as sulindac effectively prevent colon cancer in humans and rodent models. However, their cellular targets and underlying mechanisms have remained elusive. We found that dietary sulindac induced apoptosis to remove the intestinal stem cells with nuclear or phosphorylated β-catenin in *APC*$^{Min/+}$ mice. NSAIDs also induced apoptosis in human colonic polyps and effectively removed cells with aberrant Wnt signaling. Furthermore, deficiency in SMAC, a mitochondrial apoptogenic protein, attenuated the tumor-suppressive effect of sulindac in *APC*$^{Min/+}$ mice by blocking apoptosis and removal of stem cells with nuclear or phosphorylated β-catenin. These results suggest that effective chemoprevention of colon cancer by NSAIDs lies in the elimination of stem cells that are inappropriately activated by oncogenic events through induction of apoptosis.

Prevention of human cancers by using chemical agents or dietary manipulation represents a promising anticancer strategy (1, 2). Widely used nonsteroidal anti-inflammatory drugs (NSAIDs) such as sulindac and aspirin effectively prevent colon cancer in humans and rodent models (3, 4). However, their cellular targets and underlying mechanisms have remained elusive. Colorectal tumorigenesis is initiated by genetic alterations in the *APC* tumor suppressor pathway through Wnt signaling, leading to accumulation of β-catenin and its subsequent nuclear translocation (5). This process has been largely recapitulated in animal models such as *APC*$^{Min/+}$ mice, which contain an *APC* mutation and exhibit intestinal adenoma formation (6). Emerging evidence suggests that initial neoplastic proliferation in *APC*$^{Min/+}$ mice impinges upon loss of *APC* in intestinal stem cells (7, 8), including crypt base columnar (CBC) cells near the crypt bottom, as well as those located in position 4–6 (+4) counting from the crypt bottom (9). Several intestinal stem cell markers have been identified, such as Lgr5 (10), Bmi1 (8), and OLFM4 (11).

Substantial evidence indicates that the chemopreventive effects of NSAIDs are mediated by induction of apoptosis, a safeguard mechanism protecting against neoplastic transformation (12, 13). Our previous work established that NSAIDs induce mitochondria- and Bax-dependent apoptosis in colon cancer cells (14), and that SMAC (second mitochondria-derived activator of caspase), a mitochondrial apoptogenic protein (15), is an essential downstream mediator of Bax in NSAID-induced apoptosis (16, 17). In this study, we investigated the role of intestinal stem cell apoptosis in chemoprevention by NSAIDs. Our data suggest a critical role of SMAC-mediated apoptosis in removing early neoplastic stem cells in cancer chemoprevention by NSAIDs.

Results

Sulindac Treatment Induced Apoptosis in Intestinal Stem Cells of *APC*$^{Min/+}$ Mice. Dietary supplementation with NSAIDs such as sulindac for several months prevents adenoma formation in the small intestine of *APC*$^{Min/+}$ mice (18). To study the role of apoptosis in chemoprevention by NSAIDs, we first determined the time window for analyzing sulindac-induced apoptosis in *APC*$^{Min/+}$ mice because of the rapid and transient nature of apoptotic events. We found that sulindac given for only 1 wk markedly induced apoptosis detected by TUNEL staining in the small intestinal crypts of *APC*$^{Min/+}$ mice, with 22.1% of crypts containing at least one TUNEL-positive cell, compared with only 4.0% in mice receiving control diet (Fig. 1*A*). Importantly, this short exposure reduced the number of macroadenomas by 66.7% (Fig. 1*B*), consistent with observations made by others (19). Sulindac treatment for 2 wk or longer further decreased polyp numbers (Fig. S1*A*). However, TUNEL staining detected little apoptosis at 2 wk or later after treatment (Fig. 1*A* and Fig. S1*B*), suggesting that most of the apoptosis had occurred earlier. As previously shown (20), sulindac treatment did not significantly affect polyp formation in the colon of *APC*$^{Min/+}$ mice. These observations indicate that sulindac rapidly induces apoptosis in the small intestine of *APC*$^{Min/+}$ mice, and this early apoptosis may be responsible for effective chemoprevention. Therefore, 1-wk sulindac treatment was chosen for most of the subsequent experiments.

In light of recent reports that *APC* loss in intestinal stem cells efficiently promotes adenoma formation (7, 8), we further determined the types of cells undergoing apoptosis in *APC*$^{Min/+}$ mice following 1 wk of sulindac treatment. Remarkably, a majority of TUNEL-positive cells were the wedge-shaped CBC cells (62.7%) and +4 cells (27.5%), whereas apoptotic cells were rare (<10%) at higher positions in the crypts (Fig. 1 *C* and *D* and Fig. S2). Upon introducing the Lgr5-EGFP lineage marking allele (10) into *APC*$^{Min/+}$ mice, we found that sulindac treatment induced apoptosis in Lgr5-expressing cells of *Lgr5-EGFP/APC*$^{Min/+}$ mice, but not WT mice (Fig. 1 *C* and *E* and Fig. S3). The fraction of Lgr5-positive crypts containing one or more TUNEL-positive cells increased from 4.32% in the control mice to 17.60% in the sulindac-treated mice (Fig. 1*E*). We confirmed that the Lgr5-marked CBC cells and apoptotic cells at the crypt base were interspersed between MMP7-positive Paneth cells (Fig. 1*C* and Figs. S3*B* and S4) (21). Active caspase 3 staining verified the induction of apoptosis in these cells (Fig. 1*F* and Fig. S3*C*). Interestingly, apoptotic CBC cells were found to be clustered in

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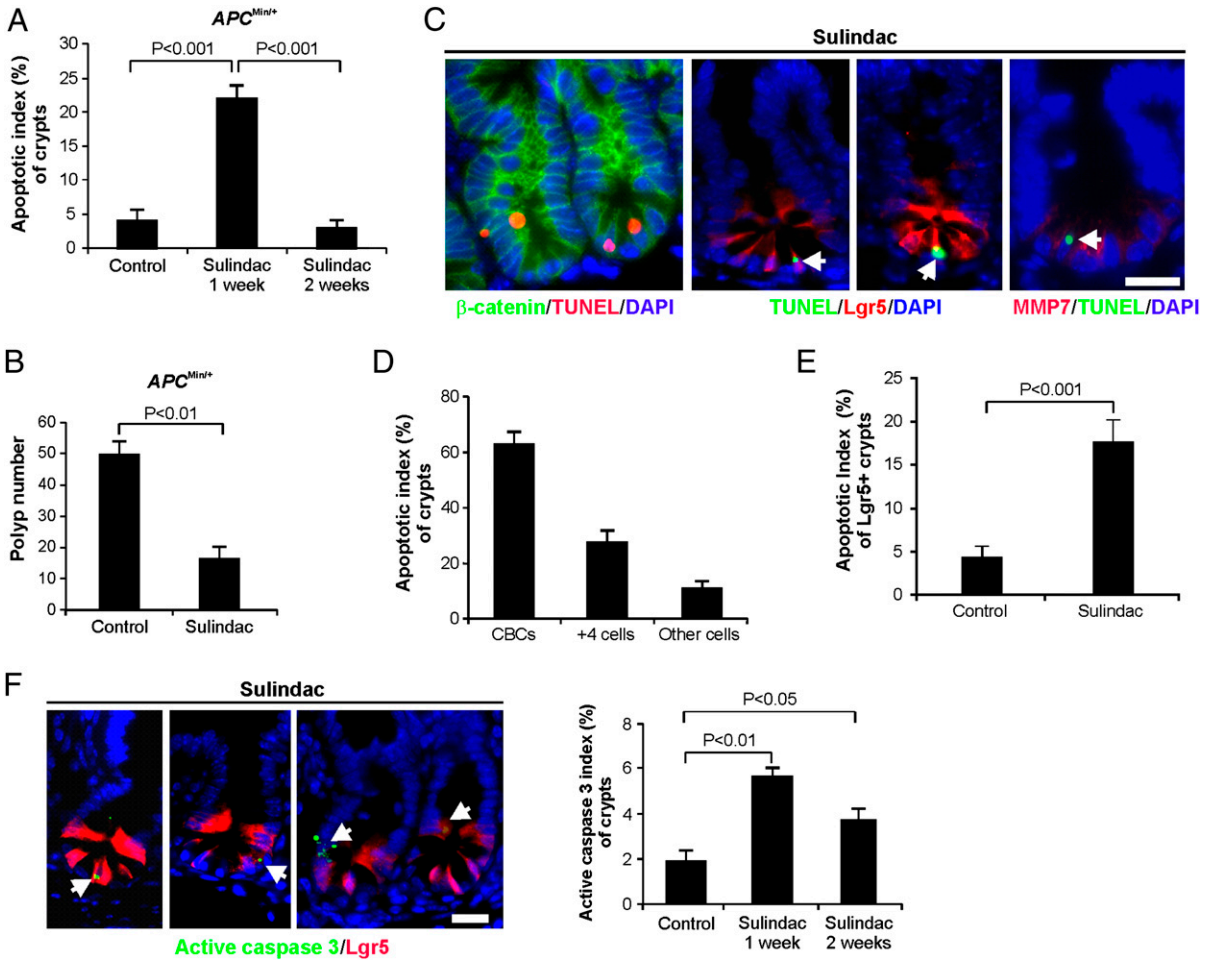

Fig. 1. Short-term sulindac administration induced apoptosis in the intestinal stem cells and suppressed adenoma formation in $APC^{Min/+}$ mice. Ten-week-old $APC^{Min/+}$ mice were fed with control or sulindac-containing (20 mg/kg/d) AIN93G diet for 1 or 2 wk and killed immediately after treatment. Intestinal polyp phenotypes, β-catenin localization, and apoptosis were analyzed. (*A*) Small intestinal sections from the treated mice were analyzed for apoptosis by TUNEL staining. The fractions of crypts containing at least one TUNEL-positive cell were determined. (*B*) Numbers of small intestinal polyps (≥0.5 mm in diameter) were counted following sulindac treatment for 1 wk. (*C*) Staining of indicated makers in $APC^{Min/+}$ mice treated with sulindac for 1 wk. For Lgr5 (EGFP) staining, $APC^{Min/+}$ mice containing the Lgr5-EGFP lineage marking allele (*Lgr5-EGFP/*$APC^{Min/+}$ mice) were analyzed. Lgr5 marks CBC cells and occasionally +4 cells, whereas MMP7 labels Paneth cells. DAPI (blue) was used for nuclear counter staining. Arrows indicate example TUNEL-positive CBCs (Lgr5-positive or MMP7-negative). (*D*) Quantification of TUNEL-positive cells based on locations in the crypts. Apoptotic index represents the fraction of crypts containing one or more TUNEL-positive cells. (*E*) Quantification of Lgr5-positive crypts containing one or more TUNEL-positive cells in *Lgr5-EGFP/*$APC^{Min/+}$ mice treated with control or sulindac diet for 1 wk. (*F*) *Left*: Staining of Lgr5 (red) and active caspase 3 (green) in $APC^{Min/+}$ mice treated with sulindac for 1 wk, with arrows indicating double positive cells. *Right*: Quantification of crypts containing one or more active caspase 3-positive cells. Values in *A*, *B*, and *D*–*F* are means ± SD (n = 6 in each group). At least 500 crypts from each animal were analyzed. (Scale bars: 15 μm.)

several neighboring Lgr5-positive crypts (Fig. 1 *C* and *F* and Fig. S3*A*), probably reflecting clonal expansion of the intestinal stem cells in which an early oncogenic event(s) occurred. These data demonstrate that intestinal stem cells are targeted for apoptosis induction following NSAID treatment.

Sulindac Treatment Removed Intestinal Stem Cells with β-Catenin Accumulation and Suppressed β-Catenin Phosphorylation. Intestinal polyp formation in $APC^{Min/+}$ mice is always accompanied by loss of the remaining WT *APC* allele (22), leading to deregulation of Wnt signaling and nuclear translocation of β-catenin (23). We therefore reasoned that sulindac may preferentially induce apoptosis in stem cells with nuclear β-catenin. Indeed, nuclear β-catenin was found in 1.92% of intestinal crypts in the control mice, including both the CBC and +4 cells, but rarely (<0.01% crypts) in other areas of the intestinal epithelium, or in the crypts of WT mice (Fig. 2*A*). Sulindac treatment for only 1 wk reduced the number of crypts containing cells with nuclear β-catenin by 75% (Fig. 2*A*). Interestingly, a vast majority (98%, 0.47%/0.48%) of identifiable CBC and +4 cells with nuclear β-catenin in sulindac-treated $APC^{Min/+}$ mice were TUNEL-positive at this time point (Fig. 2 *A* and *B*).

It has been shown that β-catenin nuclear translocation can be promoted by phosphorylation at Ser552 in the +4 cells (24). We found that the number of cells positive for β-catenin Ser552 phosphorylation (p-β-catenin), including mostly +4 and above +4 cells that did not express Lgr5 and some (11.4%) Lgr5-expressing cells (Fig. S5), was ninefold higher in $APC^{Min/+}$ mice compared with that in WT mice. Sulindac treatment significantly reduced cells with p-β-catenin (Fig. 2*C*), and induced rapid and significant apoptosis in these cells (Fig. 2*D*). These results suggest that sulindac treatment rapidly removes intestinal stem cells or progenitors with aberrant activation of Wnt signaling through induction of apoptosis.

NSAID Treatment Induced Apoptosis in Human Colonic Polyps and Removed Cells with Aberrant Wnt Signaling. To test the relevance of these observations in human patients, we analyzed colonic polyps

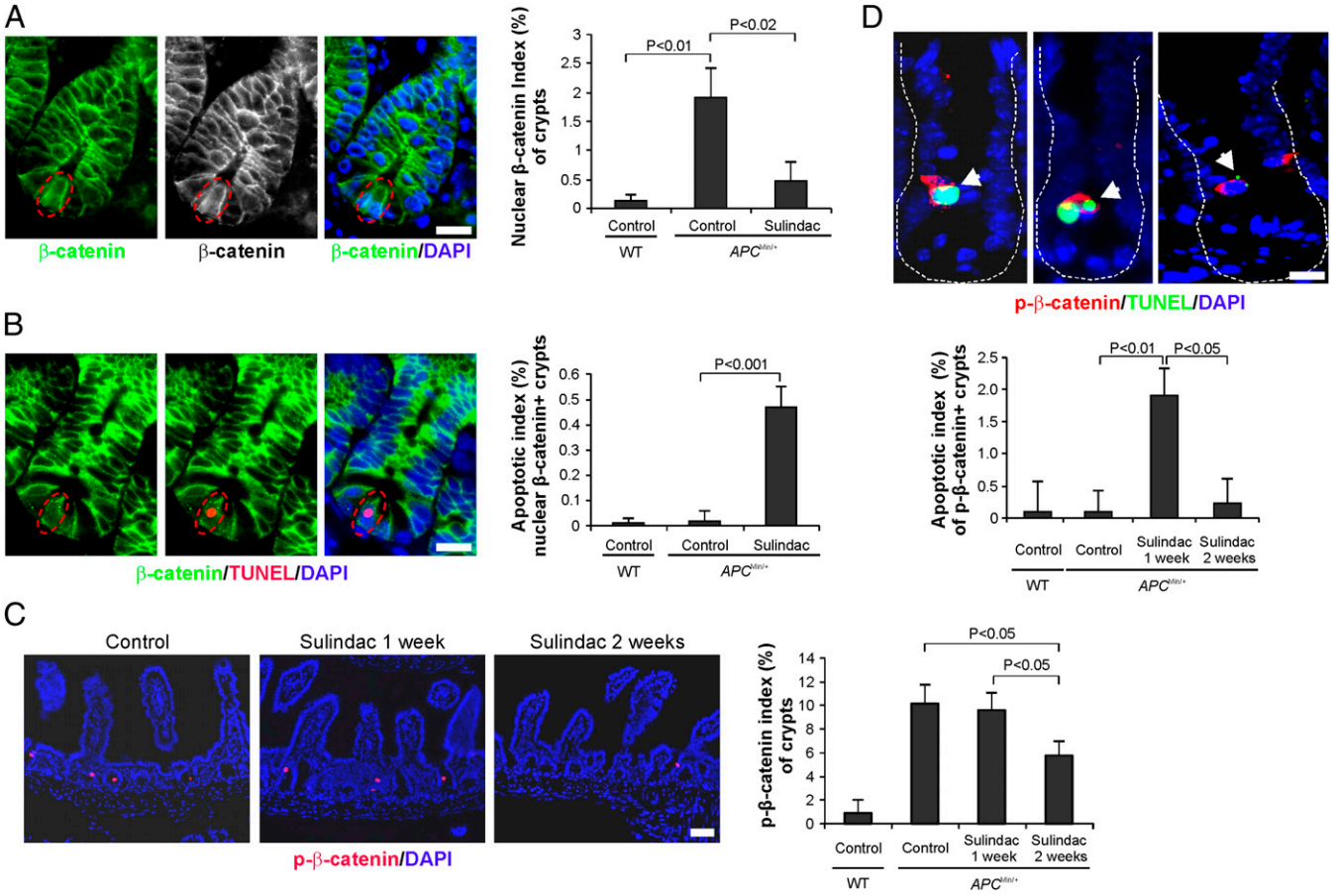

Fig. 2. Sulindac treatment removed the intestinal cells with nuclear or phospho-β-catenin via apoptosis. WT and $APC^{Min/+}$ mice were fed with control or sulindac-containing (20 mg/kg/d) diet for 1 or 2 wk and killed immediately after treatment. Small intestinal sections from the mice were analyzed for β-catenin localization, β-catenin Ser552 phosphorylation (p-β-catenin), and apoptosis (TUNEL) by immunostaining. (*A*) Analysis of β-catenin localization. *Left*: Staining of β-catenin (green or white) and DAPI (blue) in $APC^{Min/+}$ mice treated with sulindac for 1 wk. Circles mark representative CBCs with nuclear β-catenin. *Right*: Quantification of crypts with nuclear β-catenin in WT or $APC^{Min/+}$ mice treated with control or sulindac diet for 1 wk. (*B*) Analysis of β-catenin localization and apoptosis. *Left*: Staining of β-catenin (green), TUNEL (red), and DAPI (blue) in $APC^{Min/+}$ mice treated with sulindac for 1 wk. Circles mark example CBCs with nuclear β-catenin that were undergoing apoptosis. *Right*: Quantification of crypts positive for both nuclear β-catenin and TUNEL in WT and $APC^{Min/+}$ mice treated with control or sulindac diet for 1 wk. (*C*) Analysis of β-catenin phosphorylation. *Left*: Staining of p-β-catenin (red) and DAPI (blue) in $APC^{Min/+}$ mice treated with control or sulindac diet for 1 or 2 wk. *Right*: Quantification of crypts containing p-β-catenin-positive cells. (*D*) Analysis of β-catenin phosphorylation and apoptosis. *Upper*: Staining of p-β-catenin (red), TUNEL (green), and DAPI (blue) in $APC^{Min/+}$ mice treated with sulindac for 1 wk. Arrows indicate TUNEL and p-β-catenin double-positive cells. *Lower*: Quantification of crypts containing apoptotic cells in WT or $APC^{Min/+}$ mice treated with control or sulindac diet for 1 or 2 wk. Values in *A*–*D* are means ± SD ($n = 6$ in each group). At least 500 crypts from each animal were analyzed. (Scale bars: 15 μm.)

in patients taking NSAIDs. The percentage of colonic crypts containing TUNEL-positive apoptotic cells increased by more than 10-fold (from 5.04% to 51.9%) in the patients taking NSAIDs compared with those not taking NSAIDs (Fig. 3 *A* and *B* and Fig. S6). TUNEL-positive cells could be detected among those stained positive for OLFM4, a Wnt target and a CBC cell marker (11, 25) (Fig. 3*C*). Interestingly, we found that the number of p-β-catenin-positive cells decreased drastically (by more than sixfold) in patients taking NSAIDs (Fig. 3*D*). These data suggest that NSAIDs selectively induce apoptosis in human intestinal polyps with aberrant Wnt signaling.

***SMAC* Deficiency Attenuated the Chemopreventive Effect of Sulindac.** Our previous work revealed that SMAC, a mitochondrial apoptogenic protein released into cytosol during apoptosis execution (15), is essential for NSAID-induced apoptosis in colon cancer cells (16, 17). To determine whether such a mechanism operates in vivo, age- and sex-matched cohorts of $APC^{Min/+}$ mice with WT *SMAC* ($APC^{Min/+}$) or *SMAC*-KO ($SMAC^{-/-}/APC^{Min/+}$) were generated and subjected to sulindac treatment for 1 wk. *SMAC* deficiency significantly attenuated the chemopreventive effect of sulindac in $APC^{Min/+}$ mice (50.2% vs. 69.6%; $P < 0.01$; Fig. 4*A* and Fig. S7*A*). A slight increase in polyp number in *SMAC*-deficient $APC^{Min/+}$ mice was observed, and taken into the consideration. Anatomic stratification revealed that the differences were mainly in the middle and distal regions, but not in the proximal region of small intestine (Fig. 4*B*). No significant difference in polyp size was found.

***SMAC* Deficiency Impaired Sulindac-Induced Apoptosis and Suppression of Nuclear β-Catenin Accumulation.** Following 1 wk of sulindac treatment, the number of crypts with TUNEL-positive CBC/+4 cells was significantly lower in the $SMAC^{-/-}/APC^{Min/+}$ mice than in $APC^{Min/+}$ mice (9.9% vs. 22.1%; $P < 0.005$; Fig. 4*C* and Fig. S7*B*). Apoptosis in the crypts decreased significantly in both strains following 2 wk of sulindac treatment (Fig. 4*C* and Fig. S7*C*). Similarly, the number of cells or crypts with nuclear β-catenin was significantly higher in $SMAC^{-/-}/APC^{Min/+}$ mice compared with that in $APC^{Min/+}$ mice (1.33% vs. 0.48%; $P < 0.05$; Fig. 4*D*), which was correlated with a significant decrease of apoptosis in the CBC/

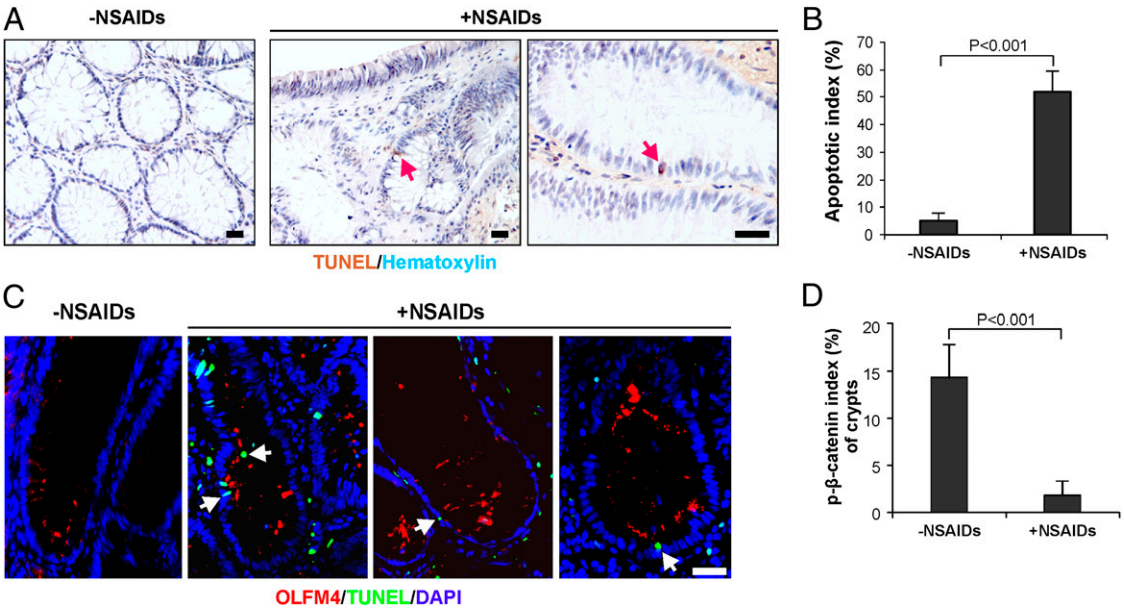

Fig. 3. NSAIDs induced apoptosis in human colonic polyps and removed cells with activated Wnt signaling. (*A*) TUNEL staining (brown) of intestinal polyps from patients taking or not taking NSAIDs. Arrows indicate TUNEL-positive apoptotic cells. (*B*) Quantification of crypts containing TUNEL-positive cells. Apoptotic index represents the percentage of intestinal crypts containing one or more TUNEL-positive cells. (*C*) Sections of intestinal polyps from four patients taking or not taking NSAIDs were stained for TUNEL (green), OLFM4 (red), and DAPI (blue). Arrows indicate TUNEL and OLFM4 double-positive cells. (*D*) Sections of intestinal polyps as in *C* were stained for p-β-catenin and quantified. Values in *B* and *D* are means ± SD ($n = 4$ in each group). At least 200 crypts from each patient were analyzed. (Scale bars: 15 μm.)

+4 cells with nuclear β-catenin (0.22% vs. 0.47%; $P < 0.05$; Fig. 4*E* and Fig. S7*B*). Furthermore, *SMAC* deficiency significantly impaired apoptosis and removal of p-β-catenin–positive cells in the crypts (Fig. 4 *F* and *G*). In addition, sulindac treatment did not affect SMAC expression in the mucosa of $APC^{Min/+}$ mice (Fig. S8*A*) and in colon cancer cells that undergo SMAC-dependent apoptosis (17) (Fig. S8*B*). These results demonstrate that SMAC-mediated apoptosis in the intestinal stem cells with aberrant activation of Wnt signaling directly contributes to chemoprevention.

Discussion

Neoplastic transformation appears to be driven by accumulation of genetic and epigenetic alterations in tissue stem cells or progenitors with pluripotency and regenerative potential (26, 27). Our results indicate that CBC and +4 intestinal stem cells accumulating nuclear or p-β-catenin are selectively removed by NSAIDs in $APC^{Min/+}$ mice through apoptosis induction, which translates into effective tumor prevention. Apoptosis in intestinal epithelial cells proceeds rapidly, typically within days (28), which may explain why we could detect sulindac-induced apoptosis only at an early time point. The partial effect of *SMAC* deficiency on sulindac-mediated chemoprevention is consistent with incomplete block of sulindac-induced apoptosis in *SMAC*-KO mice (Fig. 4*C*) and cells (17), and involvement of additional mechanisms including COX inhibition (29). The upstream events that activate Bax to trigger SMAC release following sulindac treatment remain to be delineated, and may involve death receptor signaling as suggested by several recent studies (30, 31).

Several characteristics of stem cells may explain the preferential killing of oncogenic stem cells by sulindac. Stem cells express high levels of "stemness" factors including the oncoprotein c-Myc (32), a well known apoptosis inducer (33). Therefore, stem cells with oncogenic alterations, such as loss of *APC*, may be more sensitive to NSAID-induced apoptosis, relative to differentiated cells with such alterations. It is also possible that stem cells with oncogenic alterations are simply more prevalent than differentiated cells with such alterations, because stem cells can regenerate and permanently keep acquired genetic changes, whereas differentiated cells with these changes may quickly disappear because of their rapid turnover.

Long-term use of NSAIDs, in particular COX2-specific inhibitors, is associated with side effects, which has stimulated active pursuit of new targets and combination strategies for cancer chemoprevention (34). Induction of apoptosis in oncogenic stem cells is likely to be a useful marker for successful cancer prevention, and may hold the promise for identifying novel and improved cancer chemopreventive agents. Small-molecule SMAC mimetics, which are in clinical development and can sensitize colon cancer cells to NSAID-induced apoptosis (16), may be useful as sensitizers of NSAIDs for safer and more effective cancer chemoprevention.

Methods

Mice and Treatment. All animal experiments were approved by the Institutional Animal Care and Use Committee at University of Pittsburgh. The *SMAC*-KO mice on a mixed background (129/C57BL/6) (35) were backcrossed to C57BL/6 background for 10 generations. Female $SMAC^{+/-}$ mice were crossed with $APC^{Min/+}$ mice (Jackson Laboratory) to generate $SMAC^{+/-}/APC^{Min/+}$ male mice, which were crossed to $SMAC^{+/-}$ mice to generate $APC^{Min/+}$ littermates with homozygous WT ($^{+/+}$) or null (i.e., KO; $^{-/-}$) *SMAC* alleles. The previously described *Lgr5-EGFP* (*Lgr5-EGFP*-IRES-creER^{T2}) mice (10) were crossed with $APC^{Min/+}$ mice to generate *Lgr5-EGFP*/$APC^{Min/+}$ mice. All mice were housed in micro isolator cages in a room illuminated from 7:00 AM to 7:00 PM (i.e., 12-h/12-h light-dark cycle), and allowed access to water and chow ad libitum. Genotyping was performed as previously described for *SMAC* (35) and for *Lgr5* (10). *APC* genotyping was according to the Jackson Laboratory protocol.

Treatment and Tumor Analysis. Ten-week-old and sex-matched $APC^{Min/+}$ mice with different *SMAC* and *Lgr5* genotypes were fed with control or experimental AIN93G diet (Dyets) containing 200 ppm (approximately 20 mg/kg/d) of sulindac (Sigma) for 1, 2, or 22 wk. Mice were killed immediately after treatment. Dissection of small intestine and histological analysis of adenomas (polyps; >0.5 mm in diameter) were performed as previously described (36). The adenoma counts were performed under a dissection microscope at various times following sulindac treatment.

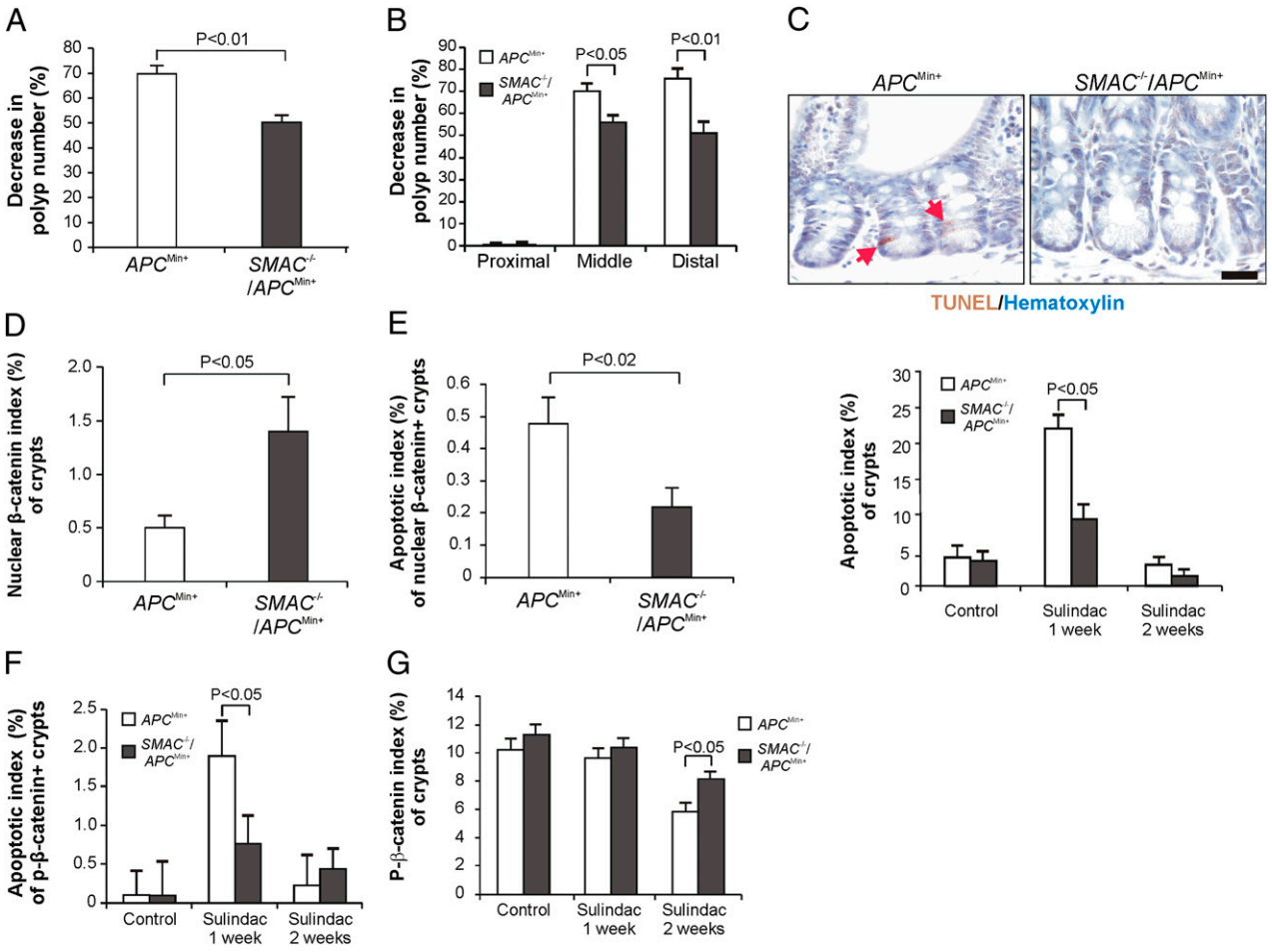

Fig. 4. *SMAC* deficiency attenuated the chemopreventive effect of sulindac in $APC^{Min/+}$ mice by blocking apoptosis in the intestinal stem cells. Age- and sex-matched parental ($APC^{Min/+}$) and *SMAC*-deficient $APC^{Min/+}$ mice ($SMAC^{-/-}/APC^{Min/+}$) were fed with control or sulindac-containing (20 mg/kg/d) diet for 1 or 2 wk and killed immediately after treatment. Intestinal polyp phenotypes, β-catenin localization, β-catenin Ser552 phosphorylation (p-β-catenin), and apoptosis were analyzed and compared. (*A*) Polyp (≥0.5 mm in diameter) number reduction in the treated mice. (*B*) Distribution of polyp number reduction in three different regions in the small intestine of the treated mice. (*C*) *Upper*: Staining of TUNEL (brown) and hematoxylin (blue) in the mice treated with sulindac for 1 wk. Arrows indicate example TUNEL-positive cells. (Scale bar: 15 μm.) *Lower*: Quantification of crypts containing one or more TUNEL-positive cells in the treated mice. (*D*) Fractions of crypts containing one or more CBC cells with nuclear β-catenin. (*E*) Fractions of crypts containing one or more TUNEL-positive cells with nuclear β-catenin. (*F*) Fractions of crypts containing one or more p-β-catenin and TUNEL double-positive cells. (*G*) Fractions of crypts containing one or more p-β-catenin–positive cells. Values are means ± SD; $n = 6$ in each group in *A*–*D*; $n = 4$ in each group in *E*–*G*. At least 500 crypts from each animal were analyzed.

Immunostaining. Tissue sections (5 μm) were deparaffinized, rehydrated, and treated with 3% hydrogen peroxide, followed by antigen retrieval in boiling 0.1 M citrate (pH 6.0) buffer for 10 min twice. The sections were then blocked by 20% goat/rabbit serum for 30 min. TUNEL staining was performed by using an ApopTag Kit (Chemicon International) according to the manufacturer's protocol. Immunostaining was performed as previously described for MMP7 (21), active caspase 3 (37), and OLFM4 (25). EGFP staining was performed at 4 °C overnight using a mouse anti-EGFP antibody (Santa Cruz Biotechnology), with Alexa 594 (Invitrogen) for signal detection. β-Catenin staining was done at 4 °C overnight using a mouse anti-β-catenin antibody (BD Biosciences), with Alexa 488 (Invitrogen) for signal detection. Staining of p-β-catenin Ser552 was performed as described (24). For double staining, TUNEL staining was performed following EGFP, β-catenin, p-β-catenin, OLFM4, or MMP-7 staining. EGFP staining was performed before MMP7, p-β-catenin, or active caspase 3 staining. Cells with positive staining were scored in at least 500 crypt sections and reported as mean ± SD.

Clinical Samples. Frozen specimens of polyps from four patients taking NSAIDs and four patients not taking NSAIDs were obtained from the Digestive Disease Tissue Resource of the University of Pittsburgh. Acquisition of tissue samples was approved by the institutional review board at the University of Pittsburgh and written informed consent was received from each patient. Paraffin blocks and sections were prepared as previously described (21) and analyzed by immunostaining.

Two male and two female subjects were represented in each category, with ages ranging from 50 to 65 y in the NSAID group and 58 to 75 y in the non-NSAID group. Subjects taking NSAIDs reported use ranging from one to three tablets per week to greater than seven tablets per week during the preceding year. The specific NSAIDs in use were not recorded. All patients had advanced adenomas by virtue of having polyps at least 1 cm in size. Four patients had tubulovillous histology and four had tubular adenomas.

Statistical Analysis. Statistical analyses were carried out using GraphPad Prism IV software. *P* values were calculated by the Student's *t* test. $P < 0.05$ was considered to be significant. The means ± 1 SD are displayed in the figures where applicable.

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