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Original Article

Oral administration of TRAIL-inducing small molecule ONC201/TIC10 prevents intestinal polyposis in the *Apc*$^{min/+}$ mouse model

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Abstract: Colorectal cancer (CRC) incidence is rising globally. Hence, preventing this disease is a high priority. With this aim, we determined the CRC prevention potential of the TRAIL-inducing small molecule ONC201/TIC10 using a preclinical model representing high-risk familial adenomatous polyposis (FAP) patients, *Apc*$^{min/+}$ mice. Prior to the efficacy study, optimal and non-toxic doses of ONC201 were determined by testing five different doses of ONC201 (0-100 mg/kg body weight (BW); twice weekly by oral gavage) in C57BL/6J mice (n=6/group) for 6 weeks. BW gain, organ weights and histopathology, blood profiling, and the plasma liver enzyme profile suggested no toxicities of ONC201 at doses up to 100 mg/kg BW. For efficacy determination, beginning at six weeks of age, groups of *Apc*$^{min/+}$ male and female mice (n≥20) treated with colon carcinogen azoxymethane (AOM) (AOM-*Apc*$^{min/+}$) were administered ONC201 (0, 25, and 50 mg/kg BW) as above up to 20 weeks of age. At termination, efficacy was determined by comparing the incidence and multiplicity of intestinal tumors between vehicle- and drug-treated groups. ONC201 showed a strong suppressive effect against the development of both large and small intestinal tumors in male and female mice. *Apc*$^{min/+}$ mice treated with ONC201 (50 mg/kg BW) showed >50% less colonic tumor incidence (P<0.0002) than controls. Colonic tumor multiplicity was also significantly reduced by 68% in male mice (0.44 ± 0.11 in treated vs. 1.4 ± 0.14 in controls; P<0.0001) and by 75% in female mice (0.30 ± 0.10 in treated vs. 1.19 ± 0.19 in controls; P<0.0003) with ONC201 treatment (50 mg/kg BW). Small intestinal polyps were reduced by 68% in male mice (11.40 ± 1.19 in treated vs. 36.08 ± 2.62 in controls; P<0.0001) and female mice (9.65 ± 1.15 in treated vs. 29.24 ± 2.51 in controls; P<0.0001). Molecular analysis of the tumors suggested an increase in TRAIL, DR5, cleaved caspases 3/7/8, Fas-associated death domain protein (FADD), and p21 (WAF1) in response to drug treatment. Serum analysis indicated a decrease in pro-inflammatory serum biomarkers, such as IL1β, IL6, TNFα, G-CSF, and GM-CSF, in the ONC201-treated mice compared with controls. Our data demonstrated excellent chemopreventive potential of orally administered ONC201 against intestinal tumorigenesis in the AOM-*Apc*$^{min/+}$ mouse model.

Keywords: Colon cancer, chemoprevention, FAP, TRAIL, ONC201, TIC10, colon polyp, min mouse

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the U.S., with ~149,000 new cases expected in 2021 [1]. More concerning are the recent trends suggesting a significant increase in CRC incidence in young adults (<50 years) and death rates in developing countries [2, 3]. The majority (>80%) of CRC cases are sporadic and are largely preventable with dietary/behavioral changes and/or effective colonoscopies [4, 5]. Despite prevention efforts and advances in treatment strategies, CRC continues to be the

second leading cause of cancer-related mortality (~50,000/year) in the U.S. [4, 6, 7], with poor 5-year survival of patients with Stage IV cancers. Hence, prevention of CRC and its recurrence in the general population, and especially in high-risk individuals with colonic polyps, and its recurrence is a high priority. Although the highest benefit in prevention can be achieved through CRC screening, the development of effective, inexpensive, and safe chemopreventive agents would greatly enhance these efforts. Thus, developing novel chemopreventive agents based on the molecular signaling of colon tumor progression may provide new chemopreventive strategies for high-risk CRC patients.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is known as a strong apoptosis inducer in many human cancer cell lines. During immune surveillance, TRAIL serves as a tumor suppressor; however, this antitumor mechanism is lost during cancer progression [8]. Thus, the TRAIL pathway is a critical effector mechanism in immune surveillance and is capable of selectively eliminating tumor cells via apoptosis without harming normal host cells [9]. TRAIL upregulated on immune cell surfaces binds to the pro-apoptotic receptors (DR4 and DR5) expressed on tumor cell surfaces to trigger the extrinsic or intrinsic cell death pathway in a cell-type-dependent manner [10]. The ability of TRAIL to initiate apoptosis selectively in cancer cells has led to clinical trials with recombinant TRAIL and the longer-lived TRAIL receptor agonist antibodies, which target either of TRAIL's two pro-apoptotic death receptors [11, 12]. Variants of recombinant TRAIL and other protein-based therapeutics continue to be developed to recapitulate the antitumor efficacy of endogenous TRAIL and to improve its stability. However, recombinant TRAIL suffers from drug properties that may limit efficacy, such as short serum half-life, stability, cost, bio-distribution, and hepatotoxicity [13]. Thus, it is important to develop novel TRAIL inducers without side effects for the chemoprevention of colon cancer.

ONC201 (TIC10) is a first-in-class, orally active small molecule with robust antitumor activity that is currently in clinical trials [11, 12, 14-16]. Studies demonstrated the safety characteristics of ONC201 in preclinical models at doses that exceed efficacious doses by 10-fold [11]. Under identical conditions, ONC201 showed selectivity on tumor cell, but not normal cell, responses; it demonstrated robust apoptotic effects in tumor cells and modest anti-proliferative effects in normal cells that were non-apoptotic and reversible. Probing the underlying mechanism of apoptosis indicated that ONC201 induces a pro-apoptotic TRAIL receptor (previously DR5) in tumor cells and is linked to its anti-tumor mechanism [11, 15]. *In vivo*, ONC201 induces TRAIL and causes potent antitumor effects when administered as a single dose (25 mg or 50 mg/kg BW) in mice with human CRC xenografts [14-16]. A phase I clinical trial with ONC201 in patients with advanced solid tumors showed no toxicity, and ONC201 was well tolerated up to 625 mg/week, up to three total doses. Micromolar plasma concentrations were achieved in these subjects after oral dosing, with concentrations that were in the preclinical therapeutic range [17]. ONC201 is currently being tested in a phase II clinical trial in subjects with select advanced solid tumor cancers. Therefore, in the present study, we performed *in vivo* bioassays to evaluate the chemopreventive efficacy of this promising agent using one of the well-recognized preclinical animal models for CRC, the *Apc*$^{min/+}$ mouse.

Methods

Agent, reagents, and diets

ONC201.2HCl was obtained from the MRI global through NCI-DCP repository for experiments performed in the Rao Lab. ONC201 was obtained from Oncoceutics/Chimerix for experiments performed in the El-Deiry Lab. PureLink genomic DNA isolation kits (Thermo Fisher cat no. K182002) were used for tail DNA preparation. Primers for genotyping were obtained from Invitrogen. Azoxymethane (AOM) was purchased from Sigma (Cat no. A5486). Diet ingredients were purchased from Bioserv or Dyets, Inc. Reagents for blood and serum enzyme profile analyses were obtained from IDEXX.

Bioassays

Animal studies were carried out at the OUHSC facilities with approval from the OUHSC IACUC.

Toxicity bioassay: To evaluate the toxicity of ONC201, six-week-old male and female C57BL/

6J mice (*n*=6/group/gender) were treated with vehicle or with five different doses of the drug (0, 12.5, 25, 50, 75, or 100 mg/kg BW) by oral gavage (twice weekly 3 days apart). After six weeks of treatment, all mice were terminated and tissues were analyzed. Major organs (livers, kidneys, spleens, etc.) were examined for any abnormalities at euthanasia and tissues were formalin-fixed for use in histological analysis. Bodyweight gain, tissue weight, histopathology, and blood profile were used for toxicity evaluation (**Figure 1**).

Pharmacodynamic biomarker: Healthy C57BL/6J mice (16 weeks age, n=11/group) were administered vehicle or ONC201 50 mg/kg body weight by gavage, twice weekly for a total of five doses and euthanized 24 hours post last gavage (Figure S1A). Colons were collected and expression of TRAIL was assayed using IHC and western immunoblotting (Figures S1B, S1C, S2).

Efficacy evaluation: *Apc*$^{min/+}$ mice for the study were generated in house by crossing male *Apc*$^{min/+}$ mice (Jax# 002020) to female C57BL/6J (jax# 000664) mice. Pups were weaned and genotyped following the established protocol [18] and enrolled in the study (*n*≥20/group/gender). To enhance colon tumor incidence, the carcinogen azoxymethane (8 mg/kg BW) was injected *s.c.* into *Apc*$^{min/+}$ mice (AOM-*Apc*$^{min/+}$) beginning at 6 weeks age, twice weekly for two weeks. Starting at 6 weeks age, ONC201 (25 and 50 mg/kg bw) was administered to experimental mice, while the placebo group was given PBS by gavage twice weekly until termination at 20 weeks of age (**Figure 2A**). All mice were fed modified AIN-76A powdered diet throughout the study. Body weights were recorded weekly in both studies. At experimental end points, mice were euthanized by CO_2 asphyxiation. For efficacy determination, colons and small intestines of *Apc*$^{min/+}$ mice were collected at necropsy. Longitudinally dissected intestines were freed of fecal contents by rinsing with PBS and tumors were evaluated for their location, size, and number to determine tumor incidence and multiplicity (**Figure 2B**).

Histopathology

Formalin-fixed tissues were processed and embedded in paraffin for sectioning. Tissue sections were prepared and stained with hematoxylin and eosin for histopathological evaluation. Blinded slides were examined by a pathologist. Kidneys, livers and colons from at least four different mice (two males and two females) from each group were evaluated for toxicity.

Blood and serum analysis

At termination, whole blood was collected by cardiac puncture and used for blood profiling and plasma preparation. Whole blood was collected into heparin-coated tubes and used for cytological analysis using the IDEXX procyte instrument, following the manufacturer's instructions. Plasma was used to analyze liver (AST, ALKP, and ALT) and kidney function (CREA and BUN) parameters using the IDEXX Catalyst instrument.

Western blotting assays

Total protein was prepared using colon tumors from control and treated mice, and concentration was determined by BCA protein assay (23225; Pierce, Rockford, IL). Protein extracts (30-60 µg protein/lane) were subjected to SDS-PAGE and electro transferred to a nitrocellulose membrane. Protein expression was detected by incubating the membranes overnight with the appropriate primary antibodies 0.5-1.0 µg/ml in Tris-buffered saline (TBS containing 0.1% Tween 20 [TBS/Tween]; Sigma). Primary antibodies used were anti-TRAIL (ab10516; 1:500), anti-DR5 (ab8416; 1:500), anti-FADD (ab24533; 1:1000), anti-caspase 3 (9662s; 1:1000), anti-caspase 7 (ab69540; 1:1000), anti-caspase 8 (ab25901; 1:1000), anti-p21 (ab109199; 1:1000), and anti-actin (sc-1616; 1:2,000). After washing, membranes were incubated with horseradish peroxidase-conjugated anti-mouse (115-035-146; 1:2000) or anti-rabbit secondary antibodies (111-035-144; 1:2000), developed using a chemiluminescence imaging system (32106; Thermo Scientific) according to the manufacturer's instructions, and quantified using GelQuant software. For pharmacodynamic biomarker study, TRAIL immunoblotting anti-TRAIL (Abcam ab231265) and anti-β-actin antibodies (Sigma-Aldrich A5441) were diluted 1:1000 in 5% non-fat milk in 1 × TTBS. Colon tissue (15 mg) from each mouse was homogenized and sonicated on ice in RIPA buffer con-

taining a protease inhibitor and samples were agitated for 2 hours at 4°C. Denaturing sample buffer was added and samples were boiled for 10 minutes. Equal amount of protein lysate was electrophoresed through 4-12% SDS-PAGE gels (Invitrogen) then transferred to PVDF membranes. The membranes were blocked with 5% non-fat milk (Sigma) in 1 × TTBS and incubated with primary antibodies in blocking buffer at 4°C overnight. Antibody binding was detected with the appropriate Pierce HRP-conjugated secondary antibody by the SynGene imaging system. Invitrogen Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP (Invitrogen #31460) and Goat anti-Mouse IgG (H+L) Secondary antibody, HRP (Invitrogen #31430) were diluted 1:5000 in 5% non-fat milk in 1 × TTBS.

Immunohistochemistry (IHC)

Paraffin sections (5-μm) of the intestinal tumors from control and treated mice were deparaffinized in xylene and rehydrated through graded ethanol solutions to phosphate-buffered saline (PBS). Heat-induced antigen retrieval (in 0.01 M citrate buffer; pH 6.0), endogenous peroxidase blocking (3% H_2O_2), and blocking were done using a standard protocol. Appropriately diluted primary antibody anti-PCNA primary antibody (ab29; 1:2000) was then applied to the tissue section and incubated overnight at 4°C, followed by washing and incubation with appropriate secondary antibody (859043; Novex Life Technologies). Slides were developed with diaminobenzidine substrate and counterstained with hematoxylin. Representative images were taken with a bright field microscope (Olympus AX71) connected to a digital imaging system. Cells with brown nuclei were considered positive. The proliferation index was determined by dividing the number of positive cells by the number of total cells and multiplying by 100.

For pharmacodynamic TRAIL staining, formalin-fixed paraffin-embedded (FFPE) tissue blocks were cut and processed as above. The sections were incubated in anti-TRAIL antibody (Abcam ab231265) diluted 1:200 in 2.5% Normal Horse Serum at 4°C overnight. Chromogenic staining was performed using DAB Substrate Kit (Vector Laboratories SK-4100) and slides were counterstained with Gill 3 Hematoxylin (Richard Allen Scientific). Secondary antibody incubation with ImmPRESS Horse Anti-Rabbit IgG Polymer Kit (Vector Laboratories MP-7401) was then used. Similarly stained negative control slides were generated by substituting the primary antibody with blocking buffer. Slides were scanned on the Leica ScanScope CS whole slide image scanner (Leica Biosystems, Buffalo Grove, Illinois, USA). Whole slide images (WSIs) were viewed at up to × 400 magnification using ImageScope (ver. 12.3.2.8013, Leica Microsystems). All digital slides were reviewed by two American Board-Certified Pathologists for the quality, and were downloaded in the SVS image format with variable degrees of JPEG image compression. WSIs were deidentified and labeled with a sticker and numbered. QuPath (version 0.2) [19] was utilized in this study as an open access solution to ML for whole slide images. All WSI files and were imported and orientated appropriately. A supervised K-nearest neighbor ML model was developed and trained from annotated patches selected from the 22 patients. No definitive training, testing, and validation cohorts were used in this study. Patches were selected to represent the tissue heterogeneity present in the data set and efforts were made to account for other staining and technical imperfections. Ignore patches covered a wide spectrum of tissue from the WSIs. Area of fat, immune cells, red blood cells, muscularis mucosa and propria, and lamina propria were included into ignore patches as well as areas of the WSI not containing tissue. Following training, the ML classifier grouped superpixels automatically based on pixel similarities between different cellular populations. In our study, this will be demonstrated as red (Positive epithelium), yellow (Negative epithelium), and black (Goblet cells) (Figure S2).

ELISA

The mouse Inflammatory Cytokines Multi-Analyte ELISArray Kit (MEM-004A; Qiagen) simultaneously analyzes a panel of 12 pro-inflammatory cytokines using an established protocol and following the manufacturer's instructions under uniform conditions in serum [18]. Results are expressed as pg/ml of plasma. Determinations were carried out in duplicate from each sample from the efficacy study.

Gene expression analysis

To study gene expression changes in response to ONC201 treatment, FFPE colon tumor tissue

slides from control and 50 mg/kg BW ONC201-treated mice (n=6/group) were processed with the HTG EdgeSeq library preparation system using the HTG EdgeSeq Mouse mRNA Tumor Response Panel (1659 genes) and sequenced on an Illumina NextSeq instrument using the standard protocol described earlier [20, 21]. Gene expression data were analyzed and data plots were generated using the HTG EdgeSeq Reveal application (3.0). Differential expression analysis was completed using the DESeq2 package (version 1.14.1). The p-value for each probe is shown after adjustment using the Benjamini and Hochberg method for controlling the false discovery rate [22].

Statistical analysis

All values are presented as Mean ± *SEM*. Differences in mean body weight gain between the treatment groups were analyzed by ANOVA. Tumor incidence was evaluated by the Fisher's Exact test. The significance of the differences in tumor multiplicity between the groups was analyzed by Student's t-test with Welch correction. The data were analyzed using GraphPad Prism 7.03 software.

Results

At the doses used, orally administered ONC201 did not cause any overt toxicity in mice

The effects of five different doses of ONC201 were evaluated in C57BL/6J mice. During the six weeks of ONC201 administration, there was no significant difference in the body weight gain between control and treatment groups in male or female mice (**Figure 1A**). All of the organs from both control and ONC201-treated animals were found to be grossly normal. For the most part, organ weights in the control and ONC201-treated groups were not significantly different in male and female mice (Tables S1 and S2). However, the average weights of livers (mg/g BW) from male mice treated with ONC201 at 12.5, 25, and 100 mg/kg BW were significantly lower than the average weights of control mouse livers (vehicle-treated); this difference was not present in the female mice. No abnormal changes were observed histologically. Only mild steatosis (fat accumulation) was found in the livers of all animals, irrespective of treatments (**Figure 1B**). Blood cytology analysis showed that ONC201 administration of up to 100 mg/kg BW did not produce any significant changes in the blood profile (Table S3). Serum parameters AST and BUN were within the normal range for mice in both control and ONC201-treated mice. While ALT, ALKP, and CREA were below the normal range for treated mice, this finding was the same for the control mice (**Figure 1C**). These results suggest no adverse effects of ONC201 within the tested dose range.

Oral ONC201-induced TRAIL expression in mouse colonic tissue

Following ONC201 administration, colonic tissue from vehicle and drug treated mice were evaluated for expression of TRAIL. Western immunoblotting (**Figure 1D**) indicated significantly increased expression of TRAIL in ONC201 treated mice compared to vehicle group. Immunohistochemistry was also performed to confirm upregulation of TRAIL in colonic mucosa, and expression levels trended higher in treated vs. control mice (**Figures 1E**, S1, S2). These data confirm TRAIL-inducing ability of ONC201 in the colonic tissue of healthy subjects following oral administration, supporting its use as a pharmacodynamic biomarker.

ONC201 reduced colon tumor incidence in $Apc^{min/+}$ mice

Based on these data, two doses of ONC201, 25 mg/kg BW (low dose) and 50 mg/kg BW (high-dose), were selected for the evaluation of preventive efficacy in the colon and small intestine (**Figure 2A** and **2B**). Intestinal tumor data indicated a strong chemopreventive effect of the ONC201 at both of the doses tested (**Figure 3**). Oral ONC201 significantly inhibited colonic and small intestinal tumors in both male and female $Apc^{min/+}$ mice. Colonic tumor incidence in $Apc^{min/+}$ male mice was dose-dependently reduced by ONC201 at 25 mg/kg (55% incidence; p<0.006; 37% inhibition) and 50 mg/kg (40% incidence; p<0.0002; 57% inhibition) doses, when compared with vehicle-treated male mice (92% incidence) (**Figure 3A**). In the $Apc^{min/+}$ female mice, significantly less colon tumor incidence was observed in mice treated with low-dose (34.8% incidence; p< 0.0027; 57% inhibition) and high-dose (30.4% incidence; P<0.001; >62% inhibition) ONC201

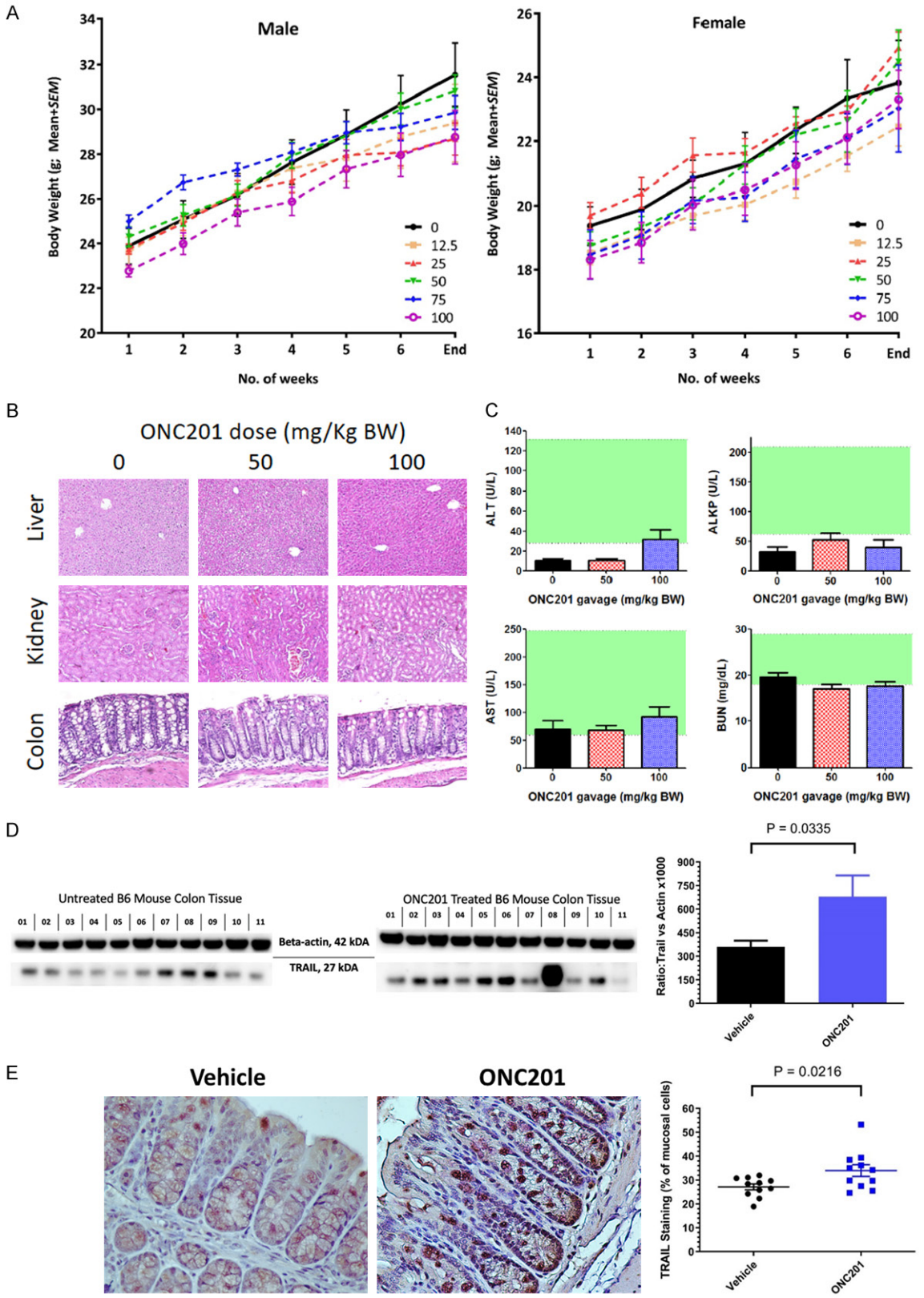

Figure 1. Toxicity evaluation of orally administered ONC201 in C57BL/6J mice. Six-week-old male and female C57BL/6J mice (*n*=6/group/gender) were treated with vehicle or with five different doses of the drug (12.5, 25, 50, 75, or 100 mg/kg BW) by oral gavage (twice weekly). After six weeks of treatment, all mice were terminated and tissues were analyzed. A. Body weight charts of the male and female mice treated with various doses of ONC201;

B. Representative histology images of liver, kidneys and colon from ONC201 treated mice; C. Serum parameters of liver and kidney toxicity; D. Western immunoblot showing TRAIL expression in colons of mice treated with vehicle and ONC201; E. Representative IHC images and quantification for ONC201 induced TRAIL expression in mouse colonic epithelium.

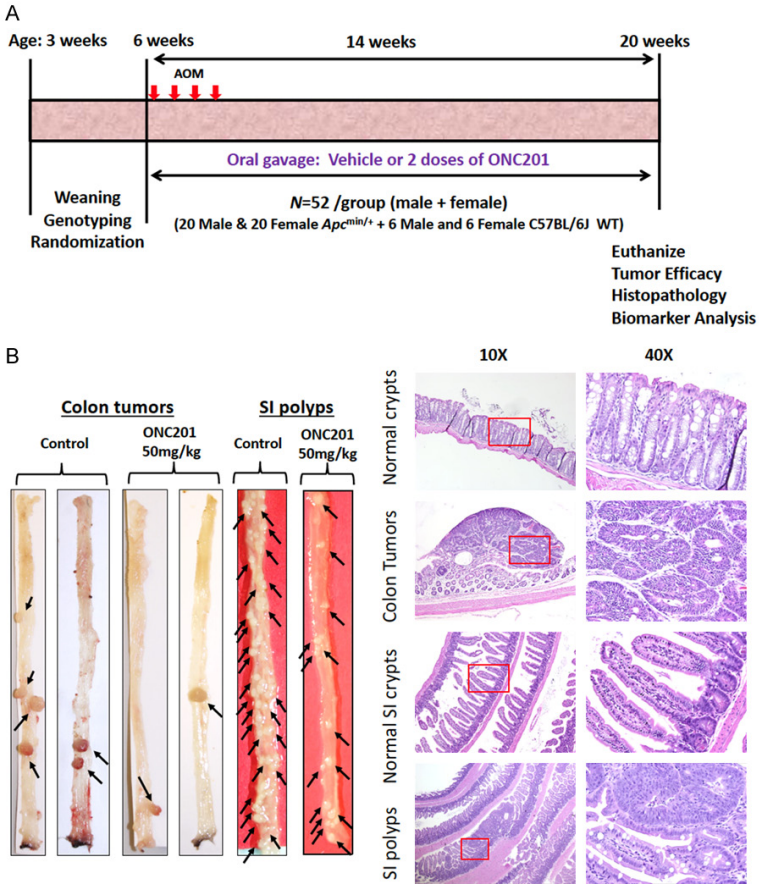

Figure 2. Bioassay design for the ONC201 chemopreventive efficacy evaluation. Six weeks old Apc$^{min/+}$ mice were enrolled in the study (n≥20/group/gender) and the carcinogen azoxymethane (8 mg/kg BW) was injected s.*c.* twice weekly for two weeks. ONC201 (25 and 50 mg/kg bw) was gavaged to experimental mice, while the vehicle group was given PBS twice weekly until termination. At 20 weeks of age, mice were euthanized by CO_2 asphyxiation, efficacy was determined by evaluation colonic and small intestinal tumors. A. Experimental design of the bioassay, B. Representative images of the intestinal tumors as observed at gross necropsy and histological evaluation.

when compared with mice treated with vehicle (81% incidence) (**Figure 3B**).

Colon tumor multiplicity is significantly reduced with ONC201 treatment

Colonic tumor multiplicity also showed a similar dose-dependent significant inhibition trend upon ONC201 treatment in the male *Apc*$^{min/+}$ mice. Male mice treated with low-dose and high-dose ONC201 had 50.1% (0.70 ± 0.16; *P*<0.001) and 68.6% (0.44 ± 0.11; *P*<0.0001) fewer colon tumors, respectively, than did the vehicle-treated male *Apc*$^{min/+}$ mice (1.4 ± 0.14) (**Figure 3C**). In the female *Apc*$^{min/+}$ mice, a non-dose-dependent, but strong, inhibition of the colon tumor multiplicity was observed. ONC201-treated female mice had up to 63% and 75% fewer colonic tumors (0.43 ± 0.14 in low dose; *P*<0.003 and 0.30 ± 0.10 in high dose; *P*<0.0003) than did vehicle-treated female mice (1.19 ± 0.19) (**Figure 3D**).

Small intestinal (SI) polyps multiplicity was strongly suppressed by ONC201 in Apc$^{min/+}$ mice

Small intestinal polyps were also significantly reduced upon treatment with ONC201 in both male and female *Apc*$^{min/+}$ mice. In the male mice, ONC201 treatment led to a 54.9% (16.25 ± 2.45; *P*<0.0001) and 68.4% (11.40 ± 1.19; *P*<0.0001) reduction in the multiplicity of SI polyps in mice treated with low and high doses of ONC201, respectively, when compared with the control group (36.08 ± 2.62) (**Figure 4A**). Similarly, female mice exhibited a 54.5% (13.30 ± 1.33; *P*<0.0001) and 67% (9.65 ± 1.15; *P*<0.0001) reduction in SI polyp multiplicity when treated with low and high doses of ONC201, respectively, and as compared with vehicle-treated mice (29.24 ± 2.51) (**Figure 4B**). Importantly, there was also a significant difference between the inhibitory effects of the low-dose and high-dose treatments in both genders, suggesting a dose-response in polyp inhibition. We also observed strong inhibition of the polyps of different sizes in treated mice (**Figure 4C** and **4D**). Formalin-

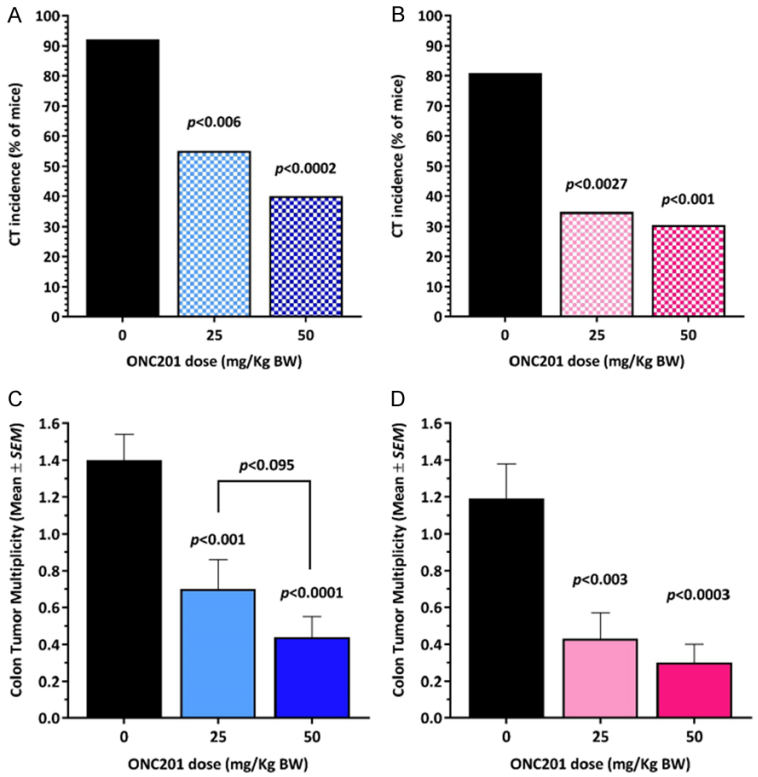

Figure 3. ONC201 treatment had significant prevent effect against colonic tumors in *Apc*$^{min/+}$ mice. Male and female *Apc*$^{min/+}$ mice were treated with ONC201 (0, 25, and 50 mg/kg bw) by oral gavage twice weekly until termination. After 14 weeks of treatment, mice were euthanized and colonic tumors were evaluated to determine efficacy. Graphs showing the colon tumor incidence in male (A) and female (B) *Apc*$^{min/+}$ mice. Graphs showing the colon tumor multiplicity in male (C) and female (D) *Apc*$^{min/+}$ mice.

fixed tumor tissues were processed for histopathological evaluation. Pathology data suggested that the colonic tumors were adenoma in all groups (**Figure 2B**). Hence, the effect of ONC201 treatment on tumor progression to carcinoma could not be determined. Overall, intestinal tumor data indicate strong and significant suppression of the colonic tumor incidence and multiplicity by ONC201, irrespective of gender. These data from the preclinical model for familial adenomatous polyposis (FAP) indicate a strong suppressive effect of the oral ONC201 against intestinal tumors and suggest that ONC201 should be further evaluated.

ONC201 treatment led to modulation of key markers of apoptosis, proliferation, and inflammation

Colonic tumors from the *Apc*$^{min/+}$ mice treated with different doses of ONC201 were evaluated for the expression of various proteins involving the TRAIL/DR5/FADD pathway and its downstream caspases. Both doses of ONC-201 induced TRAIL and DR5 in a dose-dependent manner. Only the higher dose increased the expression of FADD. An increase in cleaved fragments of caspase 7 and 8 was also observed in the high-dose-treated samples (**Figure 5A**), while cleaved caspase 3, and p21 increased in low-dose-treated samples. Immunohistochemical analysis indicated a significant decrease in the proliferation marker PCNA at both doses of ONC201 (**Figure 5B**). Circulating levels of inflammatory cytokines were also evaluated in ONC201-treated mice. Plasma samples from the control and ONC201-treated groups were analyzed for interleukins, IFN-γ, TNF-α, G-CSF, and GM-CSF. ONC201 treatment had a dose-dependent inhibitory effect on the pro-inflammatory biomarkers IL1-β, IL-6, G-CSF, and GM-CSF (**Figure 5C**).

Gene expression profile of colonic tumors was significantly altered with ONC201 treatment

Gene expression analysis of the control and ONC201-treated colon tumors using the HTG tumor panel suggested that out of 1659 genes, 846 genes were upregulated, while 813 genes were downregulated. Statistical analysis indicated 206 target genes were significantly altered in treated tumors compared with controls (101 upregulated and 105 downregulated; adjusted *p*-value <0.05) (**Figure 6**; Table S4). These genes belonged to pathways related to apoptosis, cell cycle regulation, angiogenesis, immune-oncology, and cytokines (Figure S3A-I). Among the upregulated genes were tumor suppressor genes, such as Bad, Bak1, Caspase 7, Cxcl10, Ccl17, and Gzmb, while the expression of tumor-promoting genes such as Vegfa, Brca2, Jun, and Pik3ca was downregulated (Table S4; Figure S3A-I).

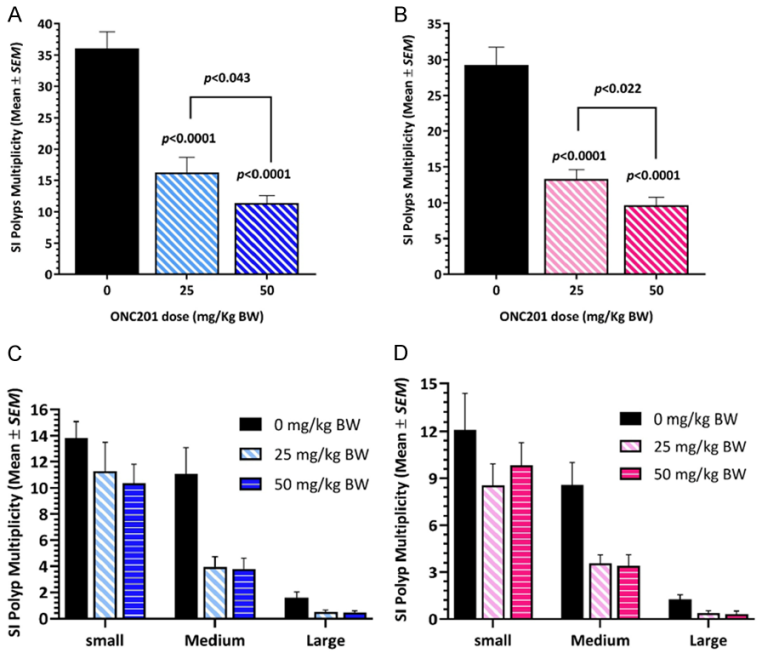

Figure 4. Effect of ONC201 on the small intestinal polyp multiplicity in male and female *Apc*$^{min/+}$ mice. Male and female *Apc*$^{min/+}$ mice were treated with ONC201 (0, 25, and 50 mg/kg bw) by oral gavage twice weekly until termination. After 14 weeks of treatment, mice were euthanized and small intestinal polyps were evaluated to determine efficacy. Graphs showing the total small intestinal polyps multiplicity in male (A) and female (B) *Apc*$^{min/+}$ mice. Graphs showing the small intestinal multiplicity by size in male (C) and female (D) *Apc*$^{min/+}$ mice. *Small <1 mm, medium 1-2 mm; Large >2 mm.*

Discussion

With 1.8 million new cases expected globally in 2021, CRC is among the top commonly diagnosed cancers in men and women [23]. High mortality rates and an increasing incidence of CRC in younger patients are a cause of major concern. While colonoscopic screening and polypectomy of adenomas can reduce CRC development, polyp recurrence and their progression to malignant tumors is common and requires continuous surveillance for further prevention. Therefore, developing alternate approaches, such as chemoprevention, can help reduce CRC incidence. TRAIL can induce apoptosis selectively in many types of cancer cells via its interaction with the death receptors [24]. Many initial attempts to target this pathway for cancer treatment using various approaches were not fully successful [25]. However, recently through high-throughput screening, ONC201 was identified as a lead small molecule p53-independent inducer of TRAIL gene transcription that rapidly underwent extensive preclinical testing, and is currently undergoing clinical evaluation [12]. Here, for the first time, we demonstrate the intestinal and colonic polyposis preventive potential of ONC201 using the *Apc*$^{min/+}$ mouse model representing FAP high-risk CRC patients.

In a six-week toxicity bioassay, we evaluated ONC201 toxicity over an approximately ten-fold dose range (12.5-100 mg/kg BW). Oral administration of ONC201 did not indicate any toxicity, as evidenced by the lack of significant differences in various indicators, such as body weights, mortality, and blood and serum profiles, between drug-treated and vehicle-treated mice. These data are consistent with previous reports indicating the excellent safety profile of ONC201 in preclinical and clinical settings [11, 12, 14, 15]. It is noteworthy that many agents, in spite of strong anticancer properties *in vitro*, fail during later stages of drug development due to unwanted side effects. Importantly, for cancer chemoprevention that requires long-term intake of preventive agents by healthy high-risk individuals, toxicity is a major concern and hurdle for clinical translatability. In this context, the oral availability and excellent safety profile of ONC201 over a wide range of doses is a desirable characteristic for use as a CRC chemopreventive agent.

In vitro, ONC201 induced TRAIL-mediated apoptotic cell death in a wide variety of human CRC cell lines with various oncogenic mutations, including APC. A similar effect was observed in *ex vivo* treatment of chemotherapy-resistant colonic tumor specimens from patients. Importantly, preclinical *in vivo* studies demonstrated the anti-cancer properties of ONC201 using CRC subcutaneous xenografts [12]. In the present efficacy evaluation study carried out in *Apc*$^{min/+}$ mice, ONC201 demonstrated a strong suppressive effect against colonic and small intestinal tumor multiplicity

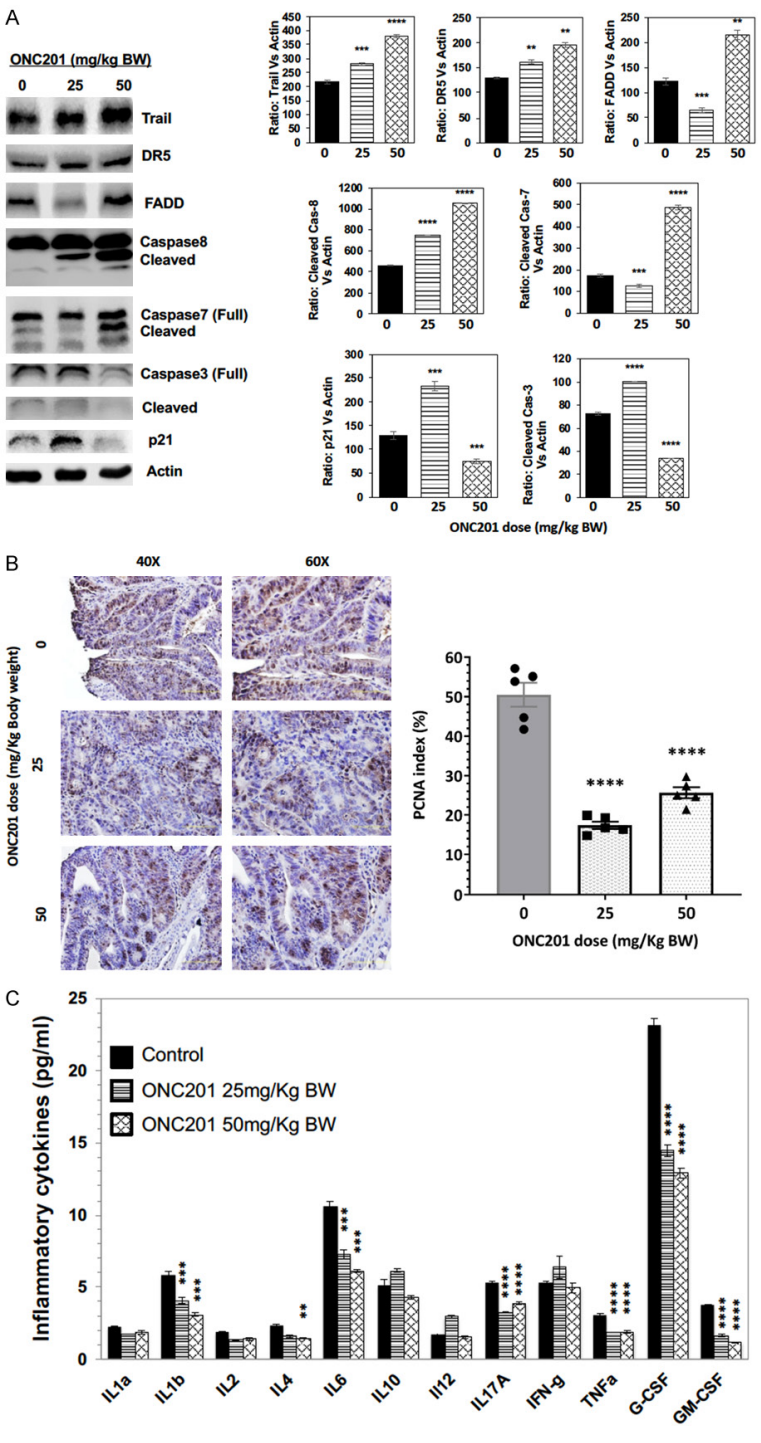

Figure 5. ONC201 induced TRAIL pathway, apoptosis markers and suppressed proliferation, and inflammation markers in treated tumors. A. Lysate from the frozen tumor samples was resolved by SDS-PAGE and changes in the expression of various proteins of TRAIL/apoptosis pathway were determined using western blotting. B. Formalin fixed tumor tissue section were analyzed for PCNA expression by immunohistochemistry. Representative IHC images used to determine the PCNA index are shown. C. Serum samples were analyzed using the mouse Inflammatory Cytokines Multi-Analyte ELISArray Kit to determine expression of 12 inflammatory cytokines.

and colonic tumor incidence in both genders. These results are consistent with the strong tumor suppressive effects observed earlier using ONC201. The observed chemopreventive effects of ONC201 in this CRC model are highly encouraging. However, the Apc mutation-driven polyposis in this model is mostly restricted to the small intestine with very few colonic tumors. Moreover, due to the short lifespan of the *Apc*$^{min/+}$ mice, tumor progression to adenocarcinoma cannot be evaluated. Therefore, further evaluation of this promising agent is warranted in other models, such as the PIRC rat, where colon tumorigenesis is similar to that observed in the high-risk FAP cohort [26]. Such a study may expedite the translation of ONC201 to clinical trials in FAP patients. Furthermore, based on the potent efficacy of ONC201, this agent should be further evaluated in models of sporadic CRC and Lynch syndrome.

Molecular analysis of the treated tumors showed induction of TRAIL pathway members along with activation of downstream effector protein, leading to apoptosis in cancer cells. In treated mice, both doses of ONC201 induced TRAIL and DR5 in a dose-dependent manner, and the higher dose produced a significant increase in FADD expression. An increase in cleaved fragments of caspase 7 and 8 was also induced by ONC201. In addition, a significant reduction of tumor cell proliferation (PCNA) was observed in ONC201-treated mouse tumors compared with

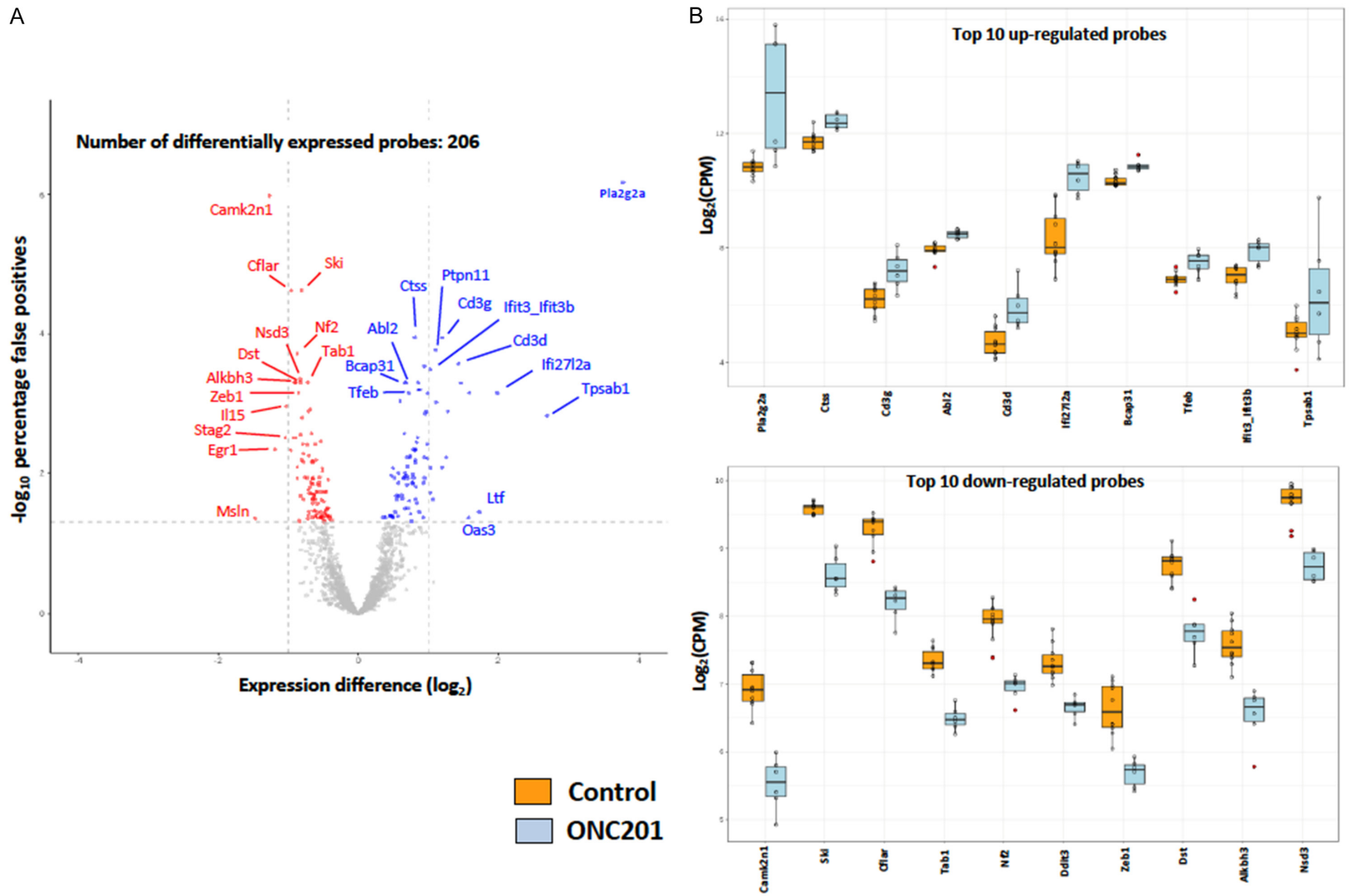

Figure 6. Gene expression analysis of the ONC201 treated tumors. FFPE colon tumor tissue slides from control and 50 mg/kg BW ONC201-treated *Apc*$^{min/+}$ mice (*n*=6/group) were processed with the HTG EdgeSeq library preparation system using the HTG EdgeSeq Mouse mRNA Tumor Response Panel (1659 genes) and sequenced on an Illumina NextSeq instrument. A. Scatter plot showing expression of various genes in the panel. B. Graphs showing the top 10 up-regulated and down-regulated genes identified.

vehicle-treated mice. These biomarker changes are consistent with previous reports [12] and correlate with tumor outcomes in the present study. Although the TRAIL-inducing effect of ONC201 is well attributed to the anti-cancer outcomes in solid tumors, recent studies in other cancers have also shown the involvement of TRAIL-independent pathways. In hematological cancers, ONC201 was shown to induce ATF4, leading to activation of mitochondrial caseinolytic protease P (ClpP) [27, 28]. Interestingly, Clpp is also overexpressed in many human cancers, including CRC [29]. Therefore, the tumor inhibitory effects of ONC201 could be due to multiple effects. Further, ONC201 treatment had an inhibitory effect on the pro-inflammatory biomarkers IL1-β, IL-6, G-CSF, and GM-CSF. Given the role of inflammation in cancer initiation and as a well-established key hallmark of cancer, these effects are noteworthy. Gene expression data indicated that ONC201 treatment led to modulation of several genes involved in apoptosis, cell cycle regulation, inflammation, angiogenesis, and immune evasion. These molecular changes agree with recent reports suggesting the potential of ONC201 to modulate the tumor microenvironment [30].

In conclusion, ONC201 demonstrated excellent efficacy against intestinal tumor development in a human-relevant mouse model that is comparable to several other targeted agents [18, 31]. These data appear highly promising and warrant further validation individually or in combination for the rapid translation to clinical trials for CRC prevention applications in individuals at risk of developing this deadly cancer.

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Disclosure of conflict of interest

W.S.E-D. is a co-founder of Oncoceutics, Inc., a subsidiary of Chimerix. Dr. El-Deiry has disclosed his relationship with Oncoceutics and potential conflict of interest to his academic institution/employer and is fully compliant with NIH and institutional policy that is managing this potential conflict of interest. Other authors have no potential conflicts of interest.

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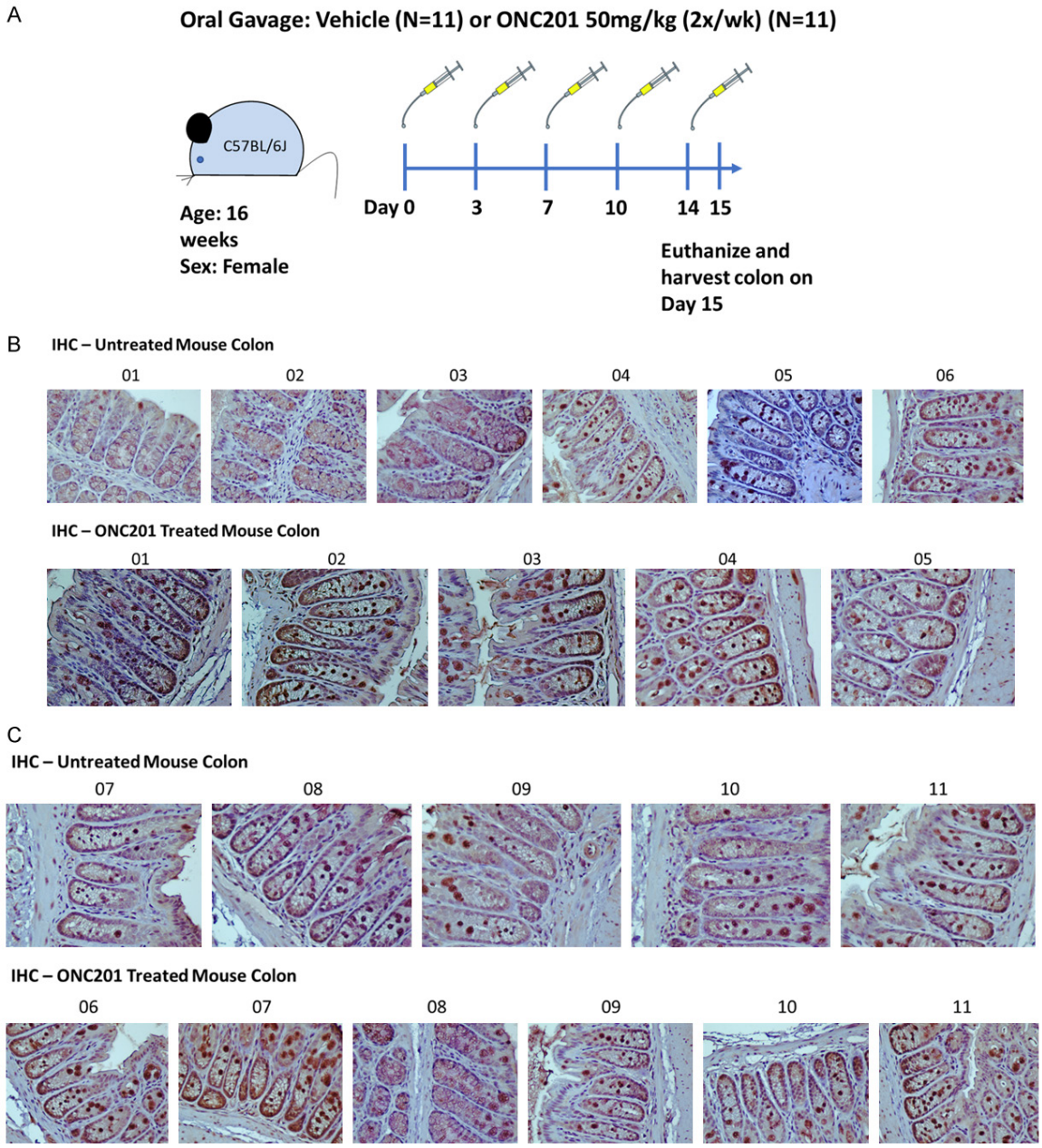


Figure S1. Female C57BL/6J mice (16 weeks age) were randomized in control and treatment groups (n=11). Mice were administered respective vehicle or ONC201 50 mg/kg body weight by gavage, twice weekly for a total of five doses. One day after last gavage all mice were euthanized and colons were collected to evaluate protein expression changes (A). Protein extracted from colonic mucosa were resolved on SDS-PAGE and expression of TRAIL was assayed using western immunoblotting. Formalin fixed colon section were analyzed using IHC for TRAIL expression (B, C).

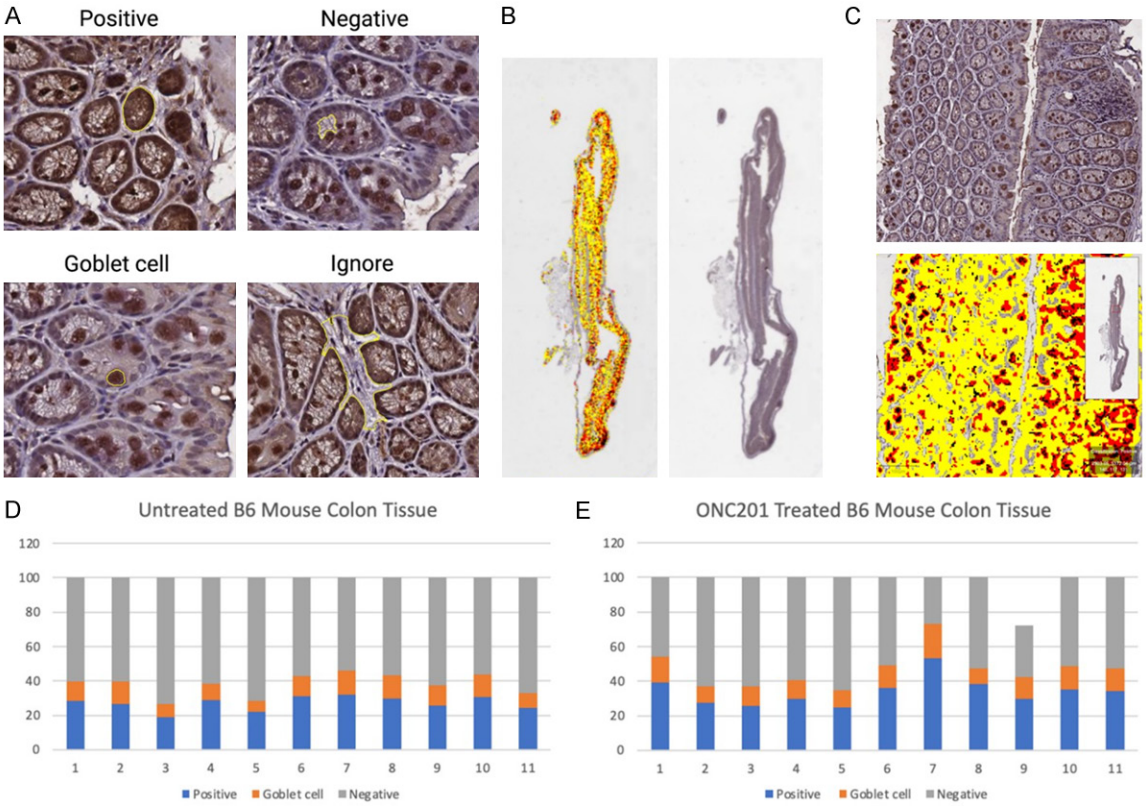


Figure S2. Female C57BL/6J mice (16 weeks age) were randomized in control and treatment groups (n=11). Mice were administered respective vehicle or ONC201 50 mg/kg body weight by gavage, twice weekly for a total of five doses. One day after last gavage all mice were euthanized and colons were collected to evaluate protein expression changes. Slides were scanned on the Leica ScanScope CS whole slide image scanner (Leica Biosystems). Whole slide images (WSIs) were collected and reviewed by two American Board-Certified Pathologists. All digital slides in the SVS image format were deidentified and quantified using QuPath (version 0.2). Patches were selected to represent the tissue heterogeneity present in the data set and efforts were made to account for other staining and technical imperfections. Area of fat, immune cells, red blood cells, muscularis mucosa and propria, and lamina propria were included into ignore patches as well as areas of the WSI not containing tissue. Following training, the ML classifier grouped superpixels automatically groups based on pixel similarities between different labeled cellular populations. A. Here this is demonstrated as red (Positive epithelium), yellow (Negative epithelium), and black (Goblet cells); B, C. Low and high magnification images of the representative IHC stained mouse colonic mucosa; D, E. Quantification of the IHC positive areas in vehicle and ONC201 treated mouse colonic mucosa.

Table S1. Organ weights (mg/g BW) after 6 weeks of ONC201 administration in male C57BL/6J mice (Mean ± *SEM*)

ONC201 (mg/kg BW)	Kidney (mg/g BW)	Liver (mg/g BW)	Spleen (mg/g BW)	Pancreas (mg/g BW)	Testis (mg/g BW)	Heart (mg/g BW)	Lungs (mg/g BW)	Colon Length (cm)
0	11.8 ± 0.4	46.9 ± 1.0	2.0 ± 0.1	7.1 ± 0.9	7.0 ± 0.4	5.0 ± 0.4	6.8 ± 0.4	7.4 ± 0.2
12.5	12.6 ± 0.5	*39.6 ± 0.7*$^{\$}$	2.1 ± 0.1	8.2 ± 1.0	6.8 ± 0.2	5.3 ± 0.5	7.4 ± 0.6	7.3 ± 0.2
25.0	12.6 ± 0.4	*38.8 ± 2.3*$^{@}$	2.4 ± 0.3	7.3 ± 0.5	7.9 ± 0.7	5.6 ± 0.2	7.1 ± 0.8	7.0 ± 0.2
50.0	11.8 ± 0.3	*40.4 ± 1.3*	1.9 ± 0.2	6.9 ± 0.5	*5.8 ± 0.3*$^{@}$	4.5 ± 0.2	*6.2 ± 0.6*$^{\#}$	7.8 ± 0.3
75.0	*14.2 ± 0.9*$^{@}$	44.2 ± 3.2	2.0 ± 0.2	8.2 ± 1.0	7.7 ± 0.4	6.3 ± 0.5	8.7 ± 0.8	7.7 ± 0.2
100.0	12.7 ± 0.9	*39.0 ± 2.7*$^{@}$	2.1 ± 0.1	6.9 ± 0.8	6.7 ± 0.5	5.7 ± 0.5	*6.3 ± 0.9*$^{@}$	7.4 ± 0.2

Significantly different from the control group, $^{@}p<0.05$; $^{\#}p<0.005$; $^{\$}p<0.0005$.

Table S2. Organ weights (mg/g BW) after 6 weeks of ONC201 administration in female C57BL/6J mice (Mean ± *SEM*)

ONC201 (mg/kg BW)	Kidney (mg/g BW)	Liver (mg/g BW)	Spleen (mg/g BW)	Pancreas (mg/g BW)	Fem. Rep (mg/g BW)	Heart (mg/g BW)	Lungs (mg/g BW)	Colon Length (cm)
0	11.6 ± 0.6	43.1 ± 1.0	2.9 ± 0.2	8.0 ± 0.9	7.7 ± 0.7	5.3 ± 0.3	8.8 ± 0.5	6.9 ± 0.1
12.5	12.1 ± 0.5	41.0 ± 1.5	2.9 ± 0.2	7.6 ± 0.8	8.2 ± 1.3	5.2 ± 0.2	9.8 ± 0.7	7.3 ± 0.2
25.0	12.5 ± 0.2	43.7 ± 3.0	3.3 ± 0.2	8.6 ± 0.9	8.9 ± 1.5	5.6 ± 0.3	9.4 ± 0.7	7.7 ± 0.3
50.0	11.0 ± 0.4	42.9 ± 1.7	4.0 ± 0.9	6.5 ± 1.0	6.9 ± 1.1	6.4 ± 0.5	9.4 ± 2.3	7.1 ± 0.2
75.0	11.0 ± 1.0	40.4 ± 1.6	4.6 ± 1.6	9.6 ± 2.3	8.9 ± 0.5	6.2 ± 0.2	*10.8 ± 1.2*$^{@}$	7.3 ± 0.2
100.0	12.5 ± 0.6	36.3 ± 6.8	3.3 ± 0.6	9.5 ± 1.0	8.9 ± 1.0	5.7 ± 0.3	9.1 ± 1.0	7.2 ± 0.1

Significantly different from the control group, $^{@}p<0.05$.

Table S3. Blood cytology profile after 6 weeks of ONC201 administration

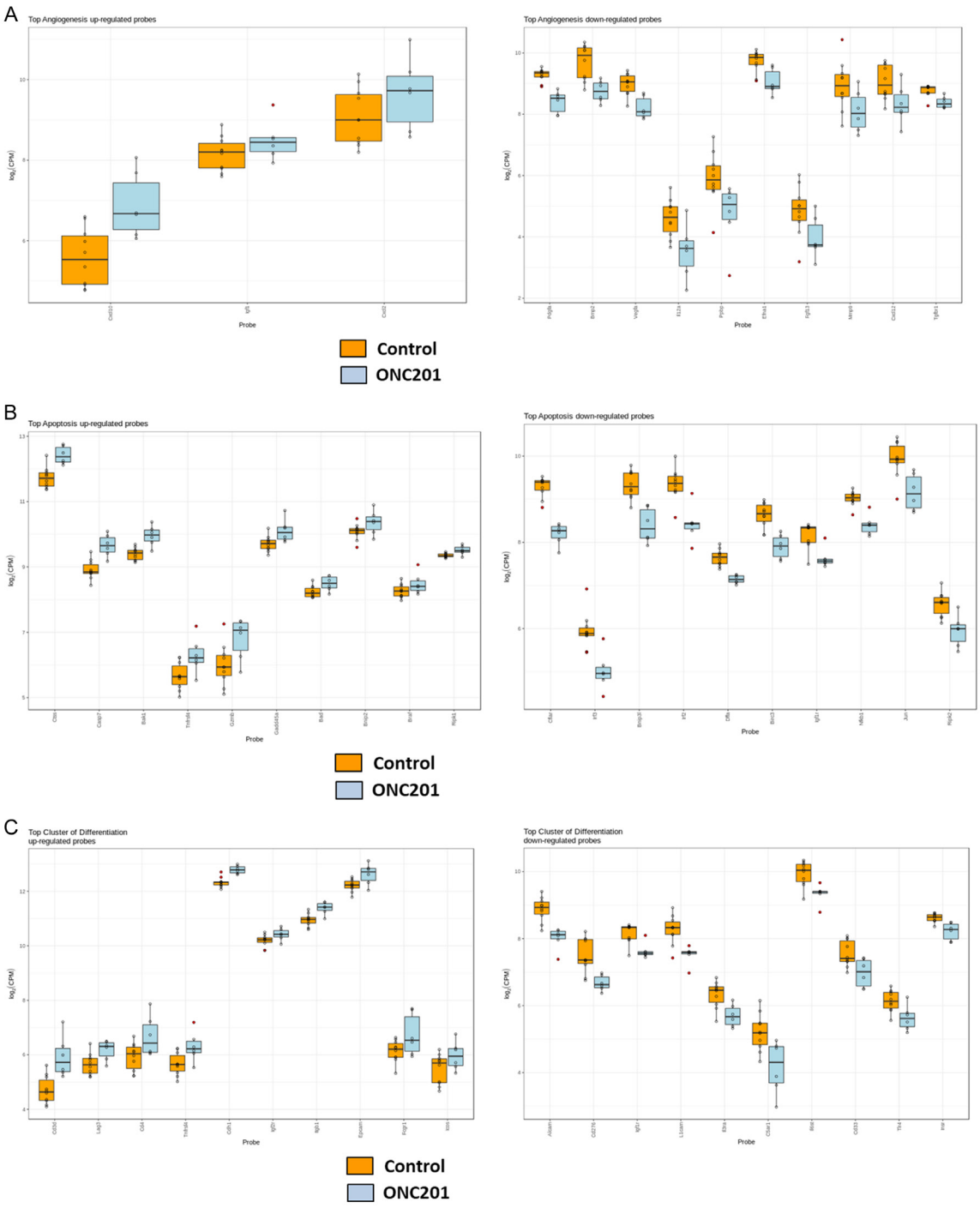
	ONC201 (mg/kg BW)					
	0	12.5	25	50	75	100
RBC (M/uL)	9.3 ± 0.3	9.7 ± 0.2	8.4 ± 0.5	9.2 ± 0.2	9.3 ± 0.2	9.2 ± 0.2
HGB (g/dL)	14.2 ± 0.5	15.3 ± 0.3	13.4 ± 0.7	14.2 ± 0.3	14.4 ± 0.2	14.7 ± 0.3
HCT (%)	47.3 ± 2.1	51.6 ± 0.2	43.9 ± 2.9	46.8 ± 0.9	48.5 ± 1.0	49.7 ± 1.2
MCV (fL)	51.0 ± 0.7	53.0 ± 0.2	52.2 ± 1.5	50.9 ± 0.3	51.9 ± 0.7	54.0 ± 1.0
MCH (pg)	15.4 ± 0.1	15.7 ± 0.1	15.9 ± 0.2	15.5 ± 0.1	15.4 ± 0.1	16.0 ± 0.5
MCHC (g/dL)	30.2 ± 0.4	29.7 ± 0.3	30.5 ± 0.7	30.4 ± 0.1	29.8 ± 0.2	29.6 ± 0.5
RET (K/uL)	371.7 ± 30.0	316.5 ± 24.4	434.7 ± 7.7	271.6 ± 2.5	303.4 ± 0.7	368.6 ± 2.2
RET% (%)	4.0 ± 0.3	3.3 ± 0.3	5.1 ± 0.5	3.0 ± 0.4	3.2 ± 0.2	4.0 ± 0.3
RBC-O (M/uL)	10.0 ± 0.5	10.4 ± 0.3	8.9 ± 0.4	9.7 ± 0.2	9.8 ± 0.1	9.5 ± 0.3
PLT (K/uL)	702.9 ± 62.3	876.5 ± 8.2	609.8 ± 6.6	801.2 ± 5.8	862.1 ± 2.4	851.0 ± 5.6
MPV (fL)	6.1 ± 0.1	6.8 ± 0.2	6.2 ± 0.1	6.0 ± 0.1	6.5 ± 0.2	6.4 ± 0.1
WBC (K/uL)	5.5 ± 0.7	5.3 ± 0.8	3.2 ± 0.4	6.3 ± 0.7	5.5 ± 0.7	6.5 ± 0.9
NEUT (K/uL)	0.9 ± 0.2	0.7 ± 0.1	0.8 ± 0.3	0.8 ± 0.1	0.7 ± 0.2	1.1 ± 0.2
LYMPH (K/uL)	4.2 ± 0.6	4.2 ± 0.7	2.8 ± 0.4	5.1 ± 0.6	4.5 ± 0.5	4.9 ± 0.7
MONO (K/uL)	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.1
EO (K/uL)	0.2 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.1
BASO (K/uL)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
NEUT% (%)	17.8 ± 2.8	14.7 ± 1.7	10.1 ± 3.2	12.5 ± 1.5	12.6 ± 3.1	17.8 ± 1.9
LYMPH% (%)	75.2 ± 3.5	77.6 ± 3.9	84.5 ± 4.2	82.0 ± 2.0	82.4 ± 3.0	74.9 ± 2.0
MONO% (%)	2.9 ± 1.4	4.0 ± 1.7	3.6 ± 1.0	1.7 ± 0.3	3.2 ± 0.6	4.3 ± 1.0
EO% (%)	3.9 ± 0.8	3.5 ± 1.1	1.5 ± 0.2	3.7 ± 0.8	1.6 ± 0.3	2.7 ± 0.5
BASO% (%)	0.1 ± 0.1	0.2 ± 0.1	0.3 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1

Table S4. Full list of genes (total 206) with significantly altered expression (101 up-regulated [Blue] and 105 down-regulated [Red] in ONC201-treated $Apc^{min/+}$ mouse colon tumors compared with untreated controls. The estimated fold change between the two groups (transformed from log-fold change) is indicated. The *p*-value for each probe is shown after adjustment using the Benjamini and Hochberg (1995) method for controlling the false discovery rate

Probe	Fold Change	adj *P*-value	Probe	Fold Change	adj *P*-value
Pla2g2a	4.10	8.03E-09	Camk2n1	-2.21	1.69E-07
Ctss	1.69	6.55E-05	Ski	-1.70	3.93E-06
Cd3g	2.04	9.75E-05	Cflar	-1.84	2.01E-05
Abl2	1.57	1.36E-04	Tab1	-1.61	4.85E-05
Cd3d	2.23	1.36E-04	Nf2	-1.75	7.62E-05
Ifi27l2a	2.55	1.56E-04	Ddit3	-1.43	1.78E-04
Bcap31	1.54	1.78E-04	Zeb1	-1.72	2.05E-04
Tfeb	1.60	1.78E-04	Dst	-1.69	2.53E-04
Ifit3_Ifit3b	1.87	2.83E-04	Alkbh3	-1.75	2.83E-04
Tpsab1	2.59	2.89E-04	Nsd3	-1.69	3.94E-04
Tapbp	1.48	2.89E-04	Fbxw11	-1.75	5.56E-04
Ptpn11	1.93	3.07E-04	Cacnb3	-1.56	6.19E-04
Hsp90b1	1.79	3.80E-04	Kmt2c	-1.58	8.84E-04
Mrc1	1.71	6.11E-04	Map3k9	-1.67	1.20E-03
Tubb5	1.71	9.27E-04	Il15	-1.83	1.80E-03
Casp7	1.72	9.27E-04	Tet2	-1.64	1.90E-03
Col5a1	1.56	1.00E-03	Svil	-1.49	2.10E-03
Cxcl10	2.14	1.00E-03	Irf3	-1.65	2.10E-03
Iigp1	1.81	1.00E-03	Ppp3r1	-1.54	2.20E-03
Lilra5	1.80	1.50E-03	Plcb4	-1.48	2.90E-03
Dtx4	1.77	1.60E-03	Ppp3cc	-1.53	3.00E-03
Rin1	1.37	2.10E-03	Tollip	-1.40	3.60E-03
Stk11	1.53	2.10E-03	Hlx	-1.62	3.60E-03
Tap1	1.78	2.30E-03	Fancf	-1.52	4.00E-03
Ifit2	1.88	2.70E-03	Atf2	-1.41	4.10E-03
Isg15	2.13	2.80E-03	Spop	-1.34	4.30E-03
Lag3	1.52	3.60E-03	Alcam	-1.58	4.30E-03
Nfkb2	1.68	4.30E-03	Ptk2	-1.43	5.00E-03
Siglec1	1.53	4.30E-03	Bnip3l	-1.62	5.30E-03
Ctsc	1.39	4.50E-03	Bmp5	-1.71	5.80E-03
Zbp1	1.95	4.50E-03	Cdkn2d	-1.37	5.80E-03
Mx2	1.69	5.00E-03	Kat2b	-1.51	6.20E-03
Mcm2	1.63	5.00E-03	Pdgfa	-1.56	6.30E-03
Epsti1	1.65	5.10E-03	Il20rb	-1.60	7.20E-03
E2f1	1.65	5.30E-03	Stag2	-1.79	7.20E-03
Cd4	1.74	6.20E-03	Rbx1	-1.35	7.90E-03
Ptpn2	1.40	7.20E-03	Shc2	-1.37	8.00E-03
Adgre5	1.58	7.20E-03	Col24a1	-1.50	1.00E-02
Bak1	1.54	7.20E-03	Irf2	-1.60	1.14E-02
Tnfaip3	1.64	7.50E-03	Klf4	-1.72	1.17E-02
Cd3e	1.61	7.90E-03	Fancc	-1.44	1.24E-02
Maff	1.58	8.00E-03	Pik3ca	-1.44	1.26E-02
Nudt13	1.51	8.00E-03	Reps1	-1.44	1.26E-02
Hist1h3b	1.63	8.10E-03	Egr1	-1.83	1.27E-02

Gpi1	1.55	8.30E-03	Cd276	-1.56	1.27E-02
Tnfrsf4	1.60	9.50E-03	Etv1	-1.66	1.31E-02
Cdh1	1.45	9.80E-03	Plce1	-1.50	1.36E-02
Ifi44	1.89	9.90E-03	Sirt4	-1.34	1.37E-02
Prdx2	1.52	1.36E-02	Npc1	-1.38	1.37E-02
Hoxa9	1.73	1.36E-02	Atr	-1.41	1.37E-02
Ddit4	1.62	1.37E-02	Dffa	-1.29	1.52E-02
Itgae	1.48	1.38E-02	Bmp2	-1.65	1.56E-02
Rsad2	1.70	1.50E-02	Notch1	-1.53	1.56E-02
Litaf	1.44	1.50E-02	Cd46	-1.47	1.56E-02
Ccl17	1.75	1.51E-02	Ythdf2	-1.34	1.71E-02
Gzmb	1.70	1.54E-02	Itga1	-1.52	1.74E-02
Ptafr	1.53	1.54E-02	Srsf2	-1.40	1.76E-02
Itgb6	1.54	1.58E-02	Brca2	-1.40	1.83E-02
Ltf	1.93	1.71E-02	Etfa	-1.50	1.91E-02
Lgals1	1.59	1.76E-02	Birc3	-1.46	1.91E-02
Igf2r	1.27	1.91E-02	Hdac1	-1.33	1.98E-02
Itgal	1.59	2.02E-02	Itga7	-1.54	2.01E-02
Clec7a	1.61	2.17E-02	Setbp1	-1.53	2.17E-02
Sfrp1	1.79	2.24E-02	Gli3	-1.43	2.17E-02
Itgb1	1.42	2.30E-02	Phlpp1	-1.35	2.30E-02
Ddx58	1.46	2.32E-02	Igf1r	-1.31	2.32E-02
Oas3	1.87	2.36E-02	Casp12	-1.49	2.36E-02
Gadd45a	1.41	2.44E-02	Ppp3ca	-1.48	2.36E-02
Epcam	1.44	2.65E-02	Rasgrp2	-1.45	2.43E-02
Sf3b1	1.35	2.70E-02	Tagap	-1.53	2.60E-02
Fcgr1	1.60	2.76E-02	Tyk2	-1.33	2.65E-02
Traf6	1.35	2.76E-02	Eif4ebp1	-1.35	2.65E-02
Elk1	1.38	2.76E-02	Mnat1	-1.27	2.65E-02
Xaf1	1.54	2.87E-02	Spry1	-1.41	2.75E-02
Il34	1.30	2.87E-02	Uty	-1.33	2.76E-02
Psma2	1.29	2.87E-02	Ubb	-1.47	2.87E-02
Crlf2	1.28	2.87E-02	Cd99	-1.31	2.87E-02
Ifit1	1.70	3.11E-02	Flna	-1.50	2.87E-02
Tmed1	1.58	3.11E-02	Map2k6	-1.39	2.87E-02
Gbp5	1.53	3.18E-02	Myb	-1.46	2.95E-02
Plcg2	1.40	3.22E-02	Vegfa	-1.48	3.00E-02
Alox15	1.71	3.31E-02	Ppp1r12b	-1.41	3.00E-02
Batf3	1.40	3.31E-02	Fut8	-1.44	3.11E-02
Abcb10	1.29	3.40E-02	Fgf9	-1.53	3.14E-02
Lig4	1.26	3.61E-02	L1cam	-1.48	3.14E-02
C1qb	1.42	3.73E-02	Nfkb1	-1.35	3.14E-02
Itgb7	1.60	3.88E-02	Wnt16	-1.60	3.21E-02
Gusb	1.64	3.88E-02	Msln	-1.80	3.33E-02
Tap2	1.33	3.88E-02	Mill2	-1.52	3.33E-02
Rad50	1.30	3.88E-02	Il3ra	-1.40	3.33E-02
Bad	1.29	3.88E-02	Nfatc4	-1.48	3.61E-02
Tmprss2	1.36	3.92E-02	Ptger3	-1.45	3.93E-02
H3f3a	1.44	3.96E-02	Tank	-1.35	3.93E-02

Stat5a	1.36	4.13E-02	Ncor1	-1.28	3.93E-02
Col4a1	1.37	4.33E-02	Lilr4b_Lilrb4a	-1.56	4.01E-02
Bcl3	1.49	4.42E-02	Anxa1	-1.58	4.06E-02
Adgre1	1.36	4.50E-02	Jun	-1.45	4.28E-02
Icos	1.46	4.75E-02	Hdac3	-1.40	4.40E-02
Ccr7	1.60	4.75E-02	S100b	-1.58	4.43E-02
Bnip2	1.33	4.89E-02	C5ar1	-1.57	4.46E-02
Alox5	1.51	4.89E-02	Atrx	-1.37	4.48E-02
			Ilf3	-1.34	4.50E-02
			Zeb2	-1.35	4.81E-02
			Sin3a	-1.36	4.89E-02
			Il12a	-1.58	4.98E-02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Probe
Control
ONC201
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log2(CPM)
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Probe
Control
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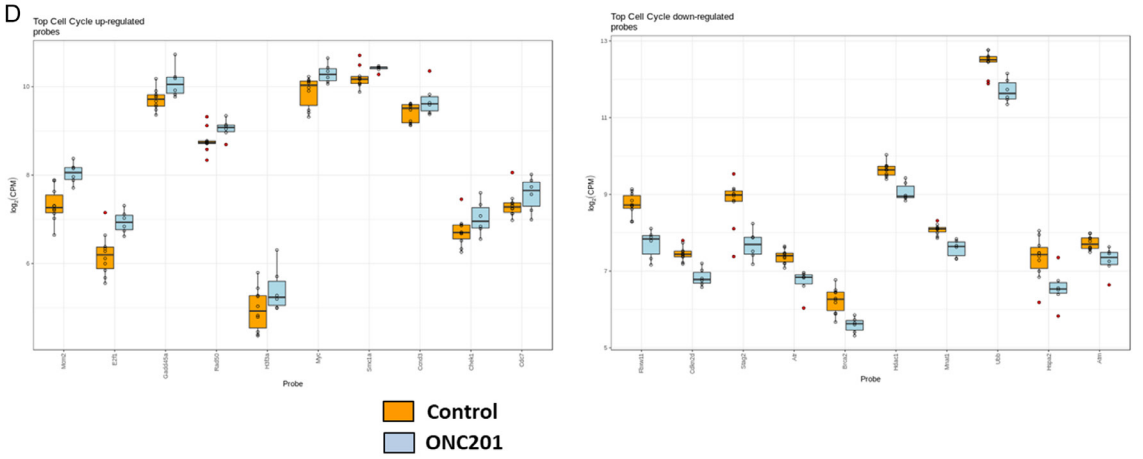
D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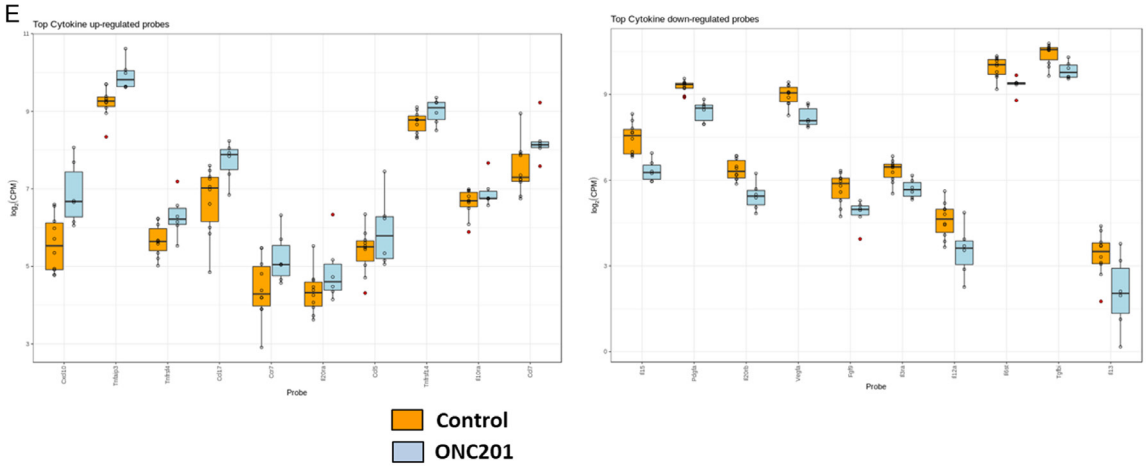
E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Probe
Control
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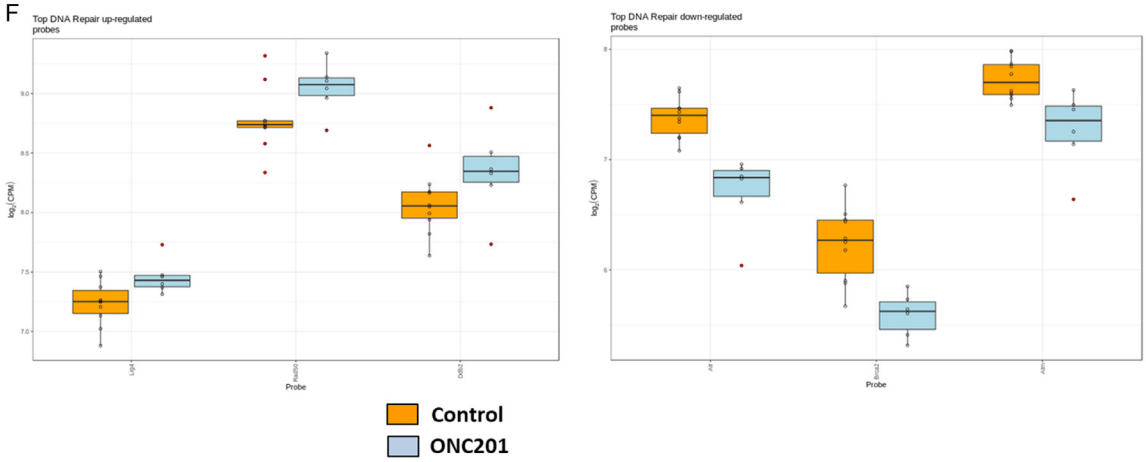
F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Probe
Control
ONC201

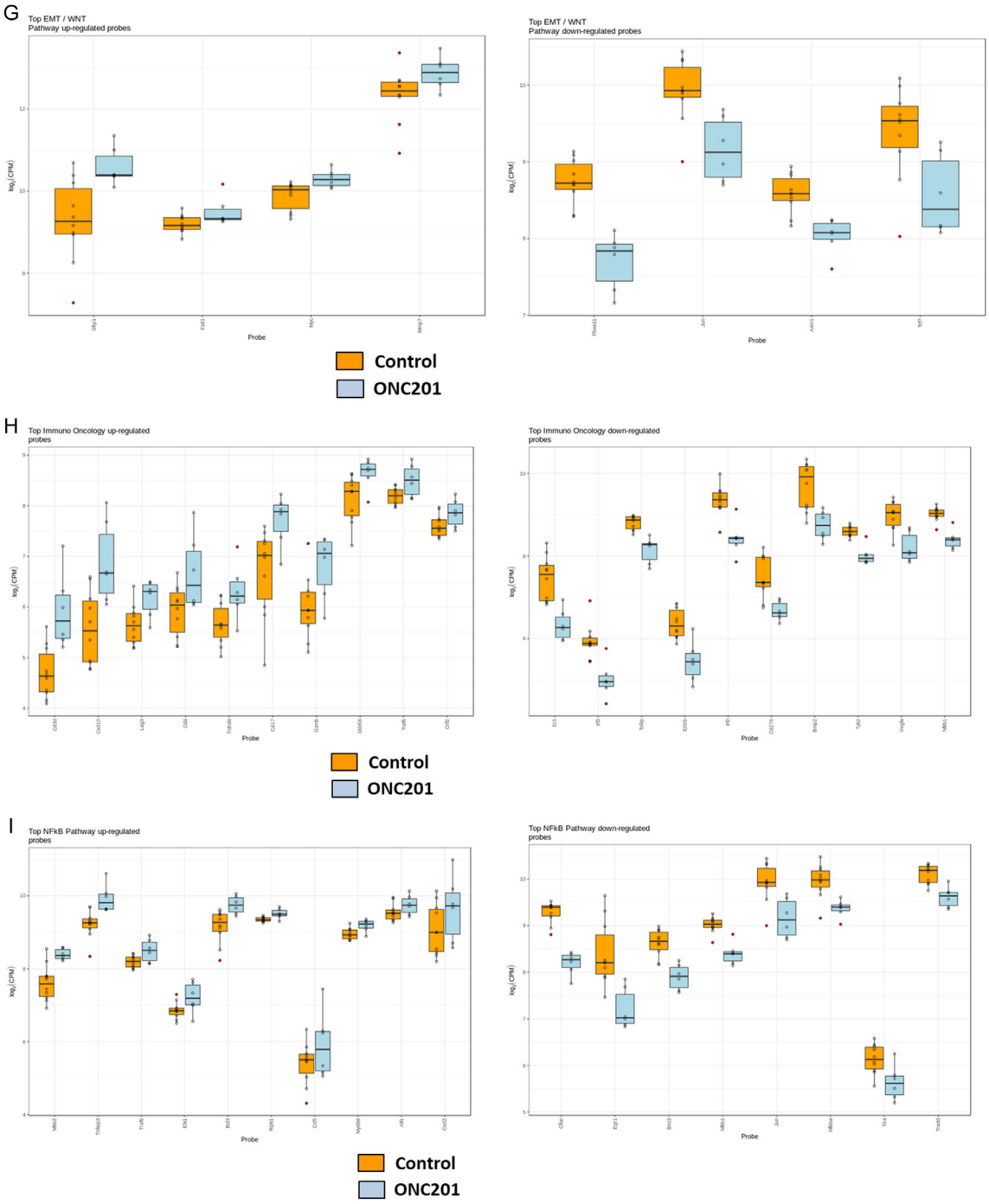

Figure S3. FFPE colon tumor tissue slides from control and 50 mg/kg BW ONC201-treated *Apc*$^{min/+}$ mice (*n*=6/group) were processed with the HTG EdgeSeq library preparation system using the HTG EdgeSeq Mouse mRNA Tumor Response Panel (1659 genes) and sequenced on an Illumina NextSeq instrument. Graphs showing genes of various pathways whose expression was either up-regulated or down-regulated in *Apc*$^{min/+}$ mouse colonic tumors in response to ONC201 treatment. Angiogenesis (A), apoptosis (B), cluster of differentiation (C), cell cycle (D), cytokines (E), DNA repair (F), EMT/WNT pathway (G), immunooncology (H), and Nfkb pathway (I).