Supplementary information

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Tubulin glycylases are required for primary cilia, control of cell proliferation and tumor development in colon
**Supplementary Materials and Methods**

**Patient samples**

Untreated human samples from colorectal adenomas (n=44), colorectal adenocarcinoma (n=59), liver metastasis (n=27) and matched normal adjacent colorectal tissues (n=136) were collected by the surgical oncology unit and stored in the Biological Resource Center (BRC) of the Institut Curie, France (Démande numéro A10-024). According to French regulations, patients were informed of research performed using the biological specimens obtained during their treatment and did not express opposition. Disease stage of CRC was based on 7th revised edition of the AJCC Colorectal Cancer.

Tissue samples were classified after into normal colon tissue, adenoma, colorectal carcinoma and liver metastasis. Normal colon tissue was identified by the maintenance of epithelial polarity, and epithelial cells with small, round, basally located nuclei. Adenomas are glandular lesions that show alterations in epithelial polarity, variable cellular atypia, as well as cellular crowding, with maintenance of the basement membrane integrity. Colorectal carcinomas are characterized by high nuclear pleomorphism, loss of nuclear polarity, numerous mitotic figures, and loss of integrity of the basement membrane. On the tissue level, carcinomas show architectural disorganization (e.g. cribriform growth pattern). Moreover, colorectal carcinoma aggressiveness is characterized by stromal invasion, lympho-vascular invasion, and necrosis. Liver metastases show a marked desmoplastic stromal response surrounding the invasive malignant glands that display comparable morphology to carcinomas found in the organ of origin.

**Animal experimentation**
Experiments were performed with C57BL/6N wild type or B6N-ttll3<sup>tm1a(EUCOMM)Wtsi</sup> strains (the latter was provided by the European Mouse Mutant Archive, EMMA). Mice were housed under conventional conditions at the animal facilities of the IGMM, and under Specific Pathogen Free (SPF) conditions in the animal facility of the Institut Curie. Animals were maintained with access to food and water <i>ad libitum</i> in a colony room kept at constant temperature (19-22°C) and humidity (40-50%) at 12-h light/dark cycles. All experimental procedures were performed in strict accordance with the guidelines of the European Community (86/609/EEC) and the French National Committee (87/848) for care and use of laboratory animals.

Mice were genotyped by polymerase chain reaction (PCR) according to EMMA protocols (EM033775) using GoTaq polymerase (Promega) and 30 amplification cycles. The following primer pairs were used to define the genotypes:

1. Ttll3_41827_F 5`-CTTGTTGATATCGTGGTATCGTTATGCACC-3` and CAS_R1_Term x 5`-CTGTCAGGAACAAAGGGGCGCCTGCCG-3`;

2. Tll3_41827_F 5`-TCTGTCAGGAACAAAGGGGCCTGGC-3` and Ttll3_41827_R 5`-CCATTGCCAGGTTGCCCAGCTGGG-3`;

3. LacZ_2_small_F 5`-GCTAATCACGACGCGCTGTA-3` and LacZ_2_small_R 5`-TTGTCAGGAACAAAGGGGCCTGGC-3`.

In this study we used the B6N-ttll3<sup>tm1a(EUCOMM)Wtsi</sup> mice carrying <i>LacZ</i> reporter cassette that is inserted into the <i>TTLL3</i> gene region. Expression of the <i>LacZ</i> gene is driven by the <i>TTLL3</i> gene promoter and allows localization of <i>TTLL3</i>-expression in tissues (Fig. 2C), while at the same time the expression of the <i>TTLL3</i> gene is disrupted (Skarnes et al, 2011; Fig. 2B). Because no detectable transcripts of <i>TTLL3</i> were found in homozygous B6N-ttll3<sup>tm1a(EUCOMM)Wtsi</sup> mice, we referred to them as “ttll3<sup>-/-</sup>” throughout this study.
To minimize the number of animals needed for our experiments, we performed key experiments in which we compared using wild type, heterozygous (TTLL3+/−) and homozygous (ttll3−/−) mice (suppl. Fig. S6). In all parameters tested, wild type and heterozygous (TTLL3+/−) behaved similar, which is why we decided to pool wild type and TTLL3+/− mice into a control group for most experiments, in particular for the AOM/DSS model.

**Histology and immunohistochemistry**

Upon sacrifice, colons were removed from the cecum to the rectum, gently flushed with PBS and fixed in formalin solution (DiaPATH F0046; MicromMicrotech, Francheville, France) for 16 h. Histological examination was performed on paraffin-embedded colon sections prepared according to “Swiss roll” procedure (Moolenbeek & Ruitenberg, 1981), and stained with Hematoxylin and Eosin (H&E), Alcian Blue, Periodic acid-Schiff (PAS) and Masson’s Trichrome for histopathological analysis (Table S2).

For immunohistochemistry using formalin-fixed and paraffin-embedded tissues were cut into 4-µm sections. Slides were deparaffinized with three 5-min washes using xylene, and pre-treated with 3% hydrogen peroxidase in PBS for 10 min. Antigen retrieval was performed using sodium citrate buffer (pH 6.0) at 95°C for 20 min in a preheated water bath. For BrdU staining, slides were treated with 2 M HCl at 37°C for 1 h, and immediately transferred to 100 mM sodium borate (pH 9.0). Blocking using PBS with 10% goat serum (G9023; Sigma-Aldrich) was performed at room temperature for 1 h. Samples were incubated with primary antibody (Table S3) for 2 h at room temperature.

For paraffin sections, samples were incubated for 1 h with 4 µg/ml of secondary HRP-coupled anti-mouse or anti-rabbit IgGs (Vector Laboratories, Burlingame, USA) in PBS at room temperature. Signals were visualized by 3,3'-Diaminobenzidine (DAB) kit (SK-4100; Vector
Laboratories, Burlingame, USA) and counterstained with Harris hematoxylin (Fisher Scientific, Fremont, USA). Fluorescence staining was also carried out on colon tissue sections fixed in 4% formaldehyde for 16-h, followed by 24 h incubation in 30% sucrose and cryoprotected with Tissue OCT compound for cryostat sectioning (Q Path 00411243; Labonord, Templemars, France). In these cases, fluorescent-labeled secondary antibodies anti-mouse/rabbit (1:1000; Molecular Probes, USA) were used. DNA was visualized by staining with 20 µg/ml 4′,6′-diamidino-2-phenylindole (DAPI). Coverslips were mounted with Prolong gold anti-fade reagent (P36930; Invitrogen, LifeTechnologies).

AOM/DSS model, tumor histology and histological grading of tumors

For tumor induction and analysis, toll3+/− and control littermate mice (7-10 weeks old) were tested for their susceptibility to develop colon tumors in an established mouse model of colitis-induced carcinogenesis. The protocol is based on administration of the mutagenic reagent azoxymethane (AOM), by intraperitoneal injection of AOM (6.25 mg/kg), followed by three cycles (each 6 days) of 2.5% (w/v) dextrane sodium sulphate (DSS) administered in the drinking water (Fig. 5A). Animals were sacrificed after 65 days, and colons were taken for histological analysis as described above.

Histological grading of AOM/DSS-induced tumors was determined with blinded genotype according to Vienna classification of gastrointestinal epithelial neoplasia (Schlemper et al, 2000). Tumors were classified as low-grade dysplasia (LGD, category 3) if they retained apico-basal nuclear orientation, goblet cell differentiation and all-round gland contours. Tumors with high-grade dysplasia (HGD, category 4.1) exhibit both architectural and cytologic changes such as loss of goblet cell differentiation, variation in nuclear size and contour and angular gland contours with focal cribriforming and focal intraglandular necrosis. Intramucosal carcinoma (IMC,
category 5.1) was characterized by extensive cribriforming, back-to-back arrangement of glands with loss of lumina and slender, irregular outpouchings consistent with early infiltrative growth (suppl. Fig. S4). A certified pathologist confirmed all our analyses.

**BrdU incorporation proliferation assay**

For proliferation studies, 12-week-old mice were intraperitoneally injected with 100 μg/g Bromodeoxyuridine (BrdU; B9285, Sigma-Aldrich) in PBS. Colons were dissected 2 h or 5 days of injection. Colons were flushed with 10 ml of cold PBS, followed by formalin solution (DiaPATH F0046; MicromMicrotech, Francheville, France). Incorporation of BrdU in proliferating intestinal epithelial cells was detected with anti-BrdU antibody (Biolegend), and the tissue was counterstained with Hematoxylin. The number of BrdU-positive cells per crypt and crypt segment was quantified of at least three animals per condition.

**β-galactosidase staining**

The *till3−/−* mouse strain contains a central targeting cassette with the reporter gene lacZ (β-galactosidase; Skarnes et al, 2011). In order to visualize the activity of the *TTLL3* promoter that drives lacZ expression, we performed 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal; Sigma-Aldrich) staining in different organs. Animals were perfused with 4% PFA in PBS and organs were cryopreserved as described at the “Histology and Immunohistochemistry” section. Colon tissue was cut to slices of 10 μm thickness. Samples were treated with 0.5% glutaraldehyde for 10 min at room temperature, followed by overnight incubation at 37°C in a staining solution containing 1 mg/ml X-gal, 5 mM potassium ferricyanide (K₃Fe(CN)₆), 5 mM potassium ferrocyanide (K₄Fe(CN)₆) and 2 mM MgCl₂, 0.1% Triton in PBS. The synthetic
substrate X-gal yields an insoluble blue precipitate when cleaved by β-galactosidase. Samples were washed in PBS for 5 min, counterstained with Nuclear Fast Red and briefly rinsed with dH₂O before mounting on aqueous mounting agent Aquatex (108562; Merck).

**Cell culture**

Cells were grown at 37°C, 5% CO₂ and 100% humidity. For long-term storage, cell lines were frozen at concentration 1x10⁶ to 2x10⁶ per ml in medium containing 50% FBS with 10% DMSO.

**Cell lines**

The cell line HEK293T, the normal human colon cell line CCD 841 CoN (ATCC® CRL-1790™) and the human carcinoma lines SW480 and DLD1 were cultured in plastic dishes and maintained in DMEM medium (41965-039; Gibco, LifeTechnologies) containing 10 μg/ml penicillin-streptomycin, 2 mM L-Glutamine, 10% fetal bovine serum (FBS; F7524; Sigma-Aldrich).

Human colon cancer cell lines SW480, SW620, DLD1, HT-29, LoVo, COLO 205, HCT116, RKO and T84 contain mutations in APC, KRAS, TP53, PIK3CA, BRAF and CTNNB1 (β-catenin) genes, which are represented in Fig. 6C (Ahmed et al, 2013; Buck et al, 2007; Rowan et al, 2000).

**Mouse embryonic fibroblasts (MEF)**

Primary mouse embryonic fibroblasts (MEF) cells were isolated from wild type or tll3⁻/⁻ mouse embryos at embryonic day 13.5 (E13.5) following an established protocol (Michalska, 2007). Heads and internal organs were surgically removed, and the remaining tissues were digested with 0.05% trypsin (25300-054; Gibco, LifeTechnologies). To obtain a single-cell suspension, the digestion mixture was pipetted up and down every 5-10 min with a 10-ml pipette for three times.
MEFs were maintained in DMEM medium containing 10 μg/ml penicillin-streptomycin, 2 mM L-glutamine, 10% FBS, 55 μM β-mercapto ethanol and 100 μM of nonessential amino acids (11140; Gibco, LifeTechnologies). MEFs were cryopreserved and stored in liquid nitrogen after isolation (passage 0). At confluence, cells were split 1:3 and could be used for four passages. For *TTLL8* silencing experiments, MEFs were cultured on coverslips until reaching confluence. The supplemented medium was replaced by starvation media (medium without serum), and cells were kept in culture for 24 h to induce cell cycle arrest and growth of primary cilia. Cells were fixed with cold methanol for 10 min at -20°C and primary cilia were visualized by immunofluorescence using anti-acetylated tubulin (6-11B-1) antibody (Table S3). MEFs were transfected with plasmids expressing shRNA against *TTLL8* and CFP (Bosch Grau et al, 2013) using JetPEI (101-40; Polyplus). 48 h after transfection, cells were fixed with cold methanol for 10 min at -20°C. MEFs were transfected with expression plasmids for shRNA and CFP. The following shRNA sequences were used:

**Control (scramble)** pHYPER-scramble-siRNA (UGCGCUACGACGACGAUG);

shRNA against *TTLL8* pHYPER-mTTLL8-siRNA_585 (GUGACCAGAUGCUAAAUCA);

shRNA against *TTLL8* pHYPER-mTTLL8-siRNA_729 (GCGAGAAGCAAGAAUUCUU);

**Primary colon epithelial cells from neonatal mice**

Colon epithelial cells (CECs) were isolated from wild type or *ttll3<−/−* mice at postnatal day 4 (P4). Colons were cut into 0.25-cm pieces and digested with 0.05% trypsin for 20 min at 37°C. The suspension was pipetted with a 10-ml pipette every 5 min to obtain single-cell suspension. At the end of the incubation, cells were filtered through a 70-μm strainer and washed with warm DMEM-F12 (1:1) medium (313331-028; Gibco, LifeTechnologies) containing 20% FBS. CECs
were plated onto 10 μg/μl fibronectin-coated coverslips and were maintained in DMEM-F12 medium containing 10 μg/ml penicillin-streptomycin and 10% FBS.

For cell cycle analysis, cells were cultured in 6-well dishes. After 48 h incubation cells were fixed with cold 70% ethanol for 2 h at -20°C. Subsequently, the DNA content was visualized by 7-aminoactinomycin D (7-AAD from BD Pharmingen, 1 μg/ml final concentration) staining using a permeabilization solution containing 0.1% Triton X-100 and 100 μg/ml RNAase in water bath at 37°C for 1 h. To assess the amount of cells in division we determined the DNA content of the cells by flow cytometry on a FACSCalibur (BD Biosciences), and determined the mitotic index (percentage of cells in phases S and M).

For induction of ciliogenesis, CECs were cultured on glass coverslips until reaching confluence. The supplemented medium was removed and cells are kept in culture for 24 h or 48 h with starvation media (medium without serum) to induce cell cycle arrest and allow the growth of primary cilia. Cells were then fixed with cold methanol for 10 min at -20°C and primary cilia were visualized by immunofluorescence using anti-detyrosinated tubulin antibodies (Table S3).

**hTTLL3 Cloning**

The sequence of human TTLL3 was only partially predicted in different databases. After prediction of a consensus sequence using the known sequence of murine TTLL3 (van Dijk et al, 2007), the gene was amplified from testis cDNA (BioChain Institute Inc., USA). The resulting DNA fragments were first cloned into the TOPO® blunt-end cloning vector (K2875-20; Invitrogen, LifeTechnologies). After isolation of single clones with inserts of ~2500 bp, these inserts were sequenced. PCR-related mutations were excluded by sequencing and comparing multiple clones. The validated fragments were subcloned into C-terminal YFP-fusion vectors. Activity tests of the exogenously expressed TTLL3 were performed by transfecting the
expression clones into cells and detecting glycylation in immunofluorescence using the monoclonal antibody TAP952.

**Microscopy and imaging**

Histological analysis were done under light microscopy and slides with tissue sections were scanned with a virtual slide Nanozoomer 2.0 HT scanner using 40x objective, and the ndpi viewer (NanoZoomer Digital Pathology, Hamamatsu, Bridgewater, NJ, USA) was used to acquire images. For fluorescent labeling, images were acquired on 3D microscope (Leica, Germany) using Metamorph software (Universal Imaging Corp., USA) or inverted Confocal SP5 (Leica, Germany) using the Leica Application Suite Advanced Fluorescence LAS AF software (Leica Microsystems, Germany). Image processing was performed using ImageJ software. For color labeling we applied Alexa fluorophore-conjugated secondary antibodies 488 (green), 568 (red) and 647 (far red). Images and quantification of ciliated cells in colon crypts (Fig. 3C) were performed using inverted Confocal SP5 microscope (Leica, Germany) and 40x oil objective. The percentage of ciliated cells was determined by counting cells bearing a primary cilium stained with antibody Arl13b. Images of ciliated CECs (Fig. 3A) were acquired using fluorescence 3D microscope (Leica, Germany) with 100x oil objective. For quantification of ciliated CECs we used 63x oil objective. The percentage of ciliated epithelial cells was determined by counting E-cadherin positive cells bearing a primary cilium stained with anti-detyrosinated tubulin antibody. Only areas of E-cadherin positive cells clusters were quantified. For quantification of ciliated MEFs we used fluorescence 3D microscope (Leica, Germany) with 63x oil objective. The percentage of ciliated MEFs was determined by cells bearing a primary cilium stained with anti-acetylated tubulin antibody. Raw images were assembled and adjusted with Adobe Photoshop.
All adjustments were done within the linear range of the gray scale gradient, and without removing minimal and maximal signals.

**RNA extraction**

Mouse organs were dissected and immediately frozen in liquid nitrogen. Tissue crushing and homogenization was done using mortar and pestles under liquid nitrogen. Tissue powders were directly transferred to an appropriate amount of TRIzol reagent (#15596-026, Life Technologies) for RNA extraction. Chloroform (0.2 ml for each 1 ml of initial TRIzol) was added to homogenized suspension and samples were vigorously shaken for 15 s. After 3 min incubation at room temperature, samples were centrifuged for 15 min at 12,000 g using a microfuge. Aqueous phase was transferred to new tubes containing isopropanol. After 15 min incubation at room temperature, samples were centrifuged for 10 min at 12,000 g and the pellet was washed with 1 ml 70% ethanol. The RNA was dissolved in 30 µl of sterile, RNAase-free water and stored at -80°C.

Concentration of RNA was determined by absorbance at 260 nm using a Nanodrop Spectrophotometer (Thermo Fisher). The quality of the RNA samples was determined by comparing the quality and relative quantities of the 18S and 28S rRNA bands after agarose-gel electrophoresis, staining with ethidium bromide, and visualization with UV light.

**Reverse transcription PCR**

For reverse transcription PCR (RT-PCR), cDNA was synthesized with First-Strand cDNA Synthesis kit according to the manufacturers instructions (27-9261-01; GE Healthcare). Briefly, we reverse-transcribed 5 µg of total RNA using the dT₁₈ primer. The use of this primer allows to specifically retro-transcribe mRNAs with poly-A tails. PCR reactions were carried out in 25 µl
containing 1x buffer (GoTaq Buffer, Promega), 0.2 mM dNTPs, 0.4 μM of each primer and 0.625 U of DNA GoTaq polymerase (Promega). PCR amplification was performed using a Thermocycler (Mastercycler Pro S, Eppendorf) and the following primers and cycling conditions:

Fig. 1A: TTLL3-F1263 (CCTGTGTAACAACTCCATCCAG) and TTLL3-R2105 (GTCATCAAGGCCTTGCTGTGG), 40 cycles;

Fig. 2B: mTTLL3_C2028-F1 (cgccgactcgagaccATGTCCCGCATGGTTCGAAATGAG; lower-case letters: part of the primer that does not hybridize with mTTLL3) and TTLL3-R855 (TATTTGGTAGTAGCGCTGAAGG), 40 cycles;

Both figures: TTLL8-F793 (CTGCCAGCATCCTCAAGTGGG) and TTLL8-R1746 (GTGGGGCTGGAGTTGATCTCG), 40 cycles.

As cDNA control, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified with GAPDH-F37 (GCACCTGGTCACCAGGGCTGC) and GAPDH-R1013 (TCCACCACCCTGTGCTGTAGCC), 20 cycles.

**Quantitative Real-time RT-PCR**

Quantitative RT-PCR was performed for the selected genes and to TBP (NM_003194 for human TBP and NM_013684 for mouse Tbp) as endogenous mRNA control. Primers are listed in Table S4, and PCR conditions are available on request. The RT-PCR protocol using the SYBR Green Master Mix kit on the ABI Prism 7900 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA) is described in detail elsewhere (Bieche et al, 1999). The relative mRNA expression levels of each gene, expressed as the N-fold difference in target gene expression relative to the TBP gene, and termed "Ntarget", was calculated as 

$$N_{target} = 2^{\Delta C_{target}}.$$  

The value of the cycle threshold (ΔCt) of a given sample was determined by subtracting the
average Ct value of the target gene from the average Ct value of the TBP gene.

**Statistical analysis**

Statistical analysis was performed by two-tailed unpaired t-test followed by Mann-Whitney post-test (Fig 1C, F; Fig 3B, D, E; Fig 4B, D, E, G; Fig 5C, D, E), and ANOVA unpaired test followed by Dunn post-test (Fig 6B) using GraphPad Prism version 5.
Supplementary Figures and Tables

Figure S1. Histological characterization of colons from wild type, TTLL3+/− and ttll3−/− mice. Paraffin-embedded colonic tissue from 10-week-old wild type, TTLL3+/− and ttll3−/− mice were stained with Hematoxylin and Eosin, Periodic acid-Schiff and Masson’s Trichrome stain to analyze colonic architecture, goblet cell population and collagens and keratins fibers content, respectively. Scale bars are 100 µm. Note that the absence of TTLL3 does not affect normal colonic histology.

Figure S2. Histological characterization of mouse colons from 16-month old wild type and ttll3−/− mice. (A) Representative images of Hematoxylin and Eosin staining of paraffin-embedded colon tissue sections of 16-month old mice, prepared as “Swiss rolls”. (B) Representative histological images of colon crypts from 16-month old mice as in (A). Scale bar is 100 µm. Note that there are no visible morphological differences between control and ttll3−/− colons.

Figure S3. Histological characterization of mouse colorectal tumors after AOM/DSS treatment. Ten-week-old control and ttll3−/− mice were submitted to the AOM/DSS protocol (Fig. 5A). Analyses were performed 65 days after initiation of the treatment. Paraffin-embedded colon tissue sections were stained. All markers analyzed show that the mouse tumors display the key characteristics of human CRC tumors. (A) Alcian blue and periodic acid-Schiff staining distinguished tumors by a reduced number of differentiated goblet cells as compared to normal epithelia. (B) Tumors were further labeled with antibodies for the proliferation marker Ki-67, combined with Hematoxylin (Scholzen & Gerdes, 2000). The staining shows a strong increase of Ki-67-positive cells in tumors. (C) Immunohistochemical staining with an anti-β-catenin
antibody combined with Hematoxylin. Tumor areas are characterized by cytoplasmic and nuclear accumulations of β-catenin, whereas adjacent healthy tissue displayed a membrane-bound distribution of β-catenin. (A, B and C) Regions with tumors and normal tissue are delineated with dotted lines. Scale bars are 200 μm. For more details on the staining methods used see Table S2.

**Figure S4. Histological classification of mouse colorectal tumors after AOM/DSS treatment.**

Representative histological images of low-grade dysplasia, high-grade dysplasia and intramucosal carcinoma in colons from AOM/DSS treated mice as used for statistical analysis in Fig. 5E. Intramucosal carcinoma was identified by the presence of intraglandular necrosis (1) and complex cribriforms (2). Paraffin-embedded tissue samples were stained with Hematoxylin and Eosin (See Material and Methods for tumor grading classification). Scale bars are 200 μm.

**Figure S5. Cloning and characterization of human TTLL3.** (A) Sequence alignment of newly cloned human and mouse TTLL3 proteins. The N-terminus of both proteins was chosen according to sequence similarity in this region. A previously cloned 5’-sequence for mouse *TTLL3*, which had no impact on the enzymatic activity of the TTLL3 protein (Rogowski et al, 2009), was not identified for human *TTLL3*. Protein domains are labeled in accordance to the previous assignment of consensus sequences (van Dijk et al, 2007). (B) Schematic representation of the domain structure of human TTLL3. (C) U2OS cells transfected with human and mouse *TTLL3-YFP* fusion genes. Cells expressing TTLL3-YFP are shown in green. Glycylated MTs are labeled with anti-monoglycylation antibody TAP952 (red), and MTs are labeled with C105 anti-tubulin polyclonal antibody (blue). Scale bars are 20 μm. (D) Expression levels of *TTLL3* analyzed by qRT-PCR in pooled cDNA samples from different human tissues. Expression was
standardized to *TATA binding protein* (*TBP*) gene.

**Figure S6. Comparative analysis of wild type, *TTLL3*<sup>+/−</sup> and *ttll3*<sup>−/−</sup> cells.** (A) Colon epithelial cells (CECs) from 4-month-old wild type, *TTLL3*<sup>+/−</sup> and *ttll3*<sup>−/−</sup> mice were cultivated for 48 h. Cells were analyzed as in Fig. 4C, D. The relative number of proliferating cells (cells in S-phase and mitosis; S + M) was plotted from wild type (n=4), *TTLL3*<sup>+/−</sup> (n=5) and *ttll3*<sup>−/−</sup> (n=6) as mean ± S.E.M. (B) Fold-increase in cell number after 48 h in culture. Mean values ± S.D. *p<10<sup>−2</sup> by two-tailed unpaired t-tests and Mann-Whitney post-test. (C) Colon epithelial cells (CECs) from *TTLL3*<sup>+/−</sup> mice at postnatal day 4 were cultured for 72-96 h and then serum-deprived for 48 h to induce cilia formation. Cells were fixed and stained with anti-E-cadherin antibody (green) to label adherens junctions at the plasma membrane, thus specifying epithelial cells. Primary cilia were visualized using anti-detyrosinated tubulin antibody (red), and nuclei were stained with DAPI (see Fig. 3D). Scale bar is 10 μm. (D) Colon epithelial cells (CECs) isolated from wild type (n=4), *TTLL3*<sup>+/−</sup> (n=3) and *ttll3*<sup>−/−</sup> (n=6) mice at postnatal day 4 were starved for 48 h to induce cilia formation. Quantification of the percentage of ciliated cells in clusters of E-cadherin-positive CECs primary culture from wild type (n=478), *TTLL3*<sup>+/−</sup> (n=381) and *ttll3*<sup>−/−</sup> (n=1183). Data are mean values between independent experiments ± S.E.M.; *p<10<sup>−2</sup> by two-tailed unpaired t-tests and Mann-Whitney post-test.
**Table S1.** Mutation profile of cell lines analyzed in Fig. 6C. Publications describing these mutations are given. The classification of the cancers is based on disease stages recommended by the American Joint Committee on Cancer (AJCC) 2010 handbook, 7th edition (collective, 2010).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin of colon cell line</th>
<th>Mutations</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCD 841 CoN</td>
<td>Normal</td>
<td>APC</td>
<td>KRAS</td>
</tr>
<tr>
<td>SW620</td>
<td>CRC stage III</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>DLD1</td>
<td>CRC stage III</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>HT-29</td>
<td>CRC stage III</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>LoVo</td>
<td>CRC stage IV</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>COLO 205</td>
<td>CRC stage IV</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>RKO</td>
<td>CRC stage IV</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>T84</td>
<td>Lung metastasis</td>
<td>x</td>
<td>x</td>
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</table>
Table S2. Specificity of staining protocols applied to histopathological characterization of mouse colons and colorectal tumors after AOM/DSS treatment.

<table>
<thead>
<tr>
<th>Staining</th>
<th>Target</th>
<th>Normal colonic mucosa</th>
<th>Colon tumor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematoxylin and Eosin</td>
<td>Nucleus (from blue to violet) and eosinophilic structures (from pink to red)</td>
<td>Cells display basally oriented nucleus not crowded and not overlapping, light red cytoplasm. Mucosa with goblet cells.</td>
<td>Position and shape nuclear alterations, cytoplasm more eosinophilic (See Material and Methods for tumor grading classification)</td>
<td>(De Robertis et al, 2011; Ponz de Leon &amp; Di Gregorio, 2001; Schlemper et al, 2000)</td>
</tr>
<tr>
<td>Alcian Blue</td>
<td>Goblet cells (bright blue)</td>
<td>Present</td>
<td>Reduced or absent</td>
<td>(Yoshimi et al, 2004)</td>
</tr>
<tr>
<td>Periodic acid-Schiff</td>
<td>Goblet cells (red)</td>
<td>Present</td>
<td>Reduced or absent</td>
<td>(An et al, 2007)</td>
</tr>
<tr>
<td>Masson’s Trichrome</td>
<td>Keratin and muscle fibers (red), collagen (blue or green)</td>
<td>Present</td>
<td>Increased: deposition of collagen fibrils</td>
<td>(Halberg et al, 2000)</td>
</tr>
<tr>
<td>Ki-67</td>
<td>Proliferating cells (brown)</td>
<td>Base of the crypt</td>
<td>Scattered throughout the crypt</td>
<td>(Johnston et al, 1989)</td>
</tr>
<tr>
<td>β-catenin</td>
<td>β-catenin localization (brown)</td>
<td>Membrane</td>
<td>Cytoplasm and nucleus</td>
<td>(Iwamoto et al, 2000; Sheng et al, 1998)</td>
</tr>
</tbody>
</table>

Table S3. Primary antibodies used in this study

<table>
<thead>
<tr>
<th>Antibody name</th>
<th>Reactivity</th>
<th>Dilution</th>
<th>Provider</th>
</tr>
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<tbody>
<tr>
<td>anti-β-catenin (E247)</td>
<td>rabbit</td>
<td>1:200</td>
<td>Abcam #ab32572</td>
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<td>anti-cyclin D1 (92G2)</td>
<td>rabbit</td>
<td>1:50</td>
<td>Cell Signaling #2978</td>
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<td>TAP952 (anti-monoglycylated tubulin)</td>
<td>mouse</td>
<td>1:200</td>
<td>gift of A. Aubusson-Fleury</td>
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<td>anti-acetylated tubulin (clone 6-11B-1)</td>
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<td>1:1000</td>
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<td>anti-Ki-67</td>
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<td>LabVision #RB9043P</td>
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Table S4. Primers for qRT-PCR

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<th>Gene</th>
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<th>Reverse</th>
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<td>TTLL3</td>
<td>human</td>
<td>5’-GATGCTGGACCAGCAACTGTGA-3’</td>
<td>5’-GCTTCTTGATGGTGAGCCTCTC-3’</td>
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<tr>
<td>TTLL8</td>
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<td>5’-ACTCCCTCGGATGCGAT-3’</td>
<td>5’-TGAGGTCCACAGAGTGACTCA-3’</td>
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<tr>
<td>TBP</td>
<td>human</td>
<td>5’-TGCAACAGGACCAAGTGGA-3’</td>
<td>5’-CACATCAAGCTCCCCACCA-3’</td>
</tr>
<tr>
<td>TTLL3</td>
<td>mouse</td>
<td>5’-AGCGGACTGGACACAGAGA-3’</td>
<td>5’-GAATGGACTGGAGAATGGA-3’</td>
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<tr>
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<td>5’-CTCCCTTTATCTCTTGGCCTTGACT-3’</td>
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<tr>
<td>TBP</td>
<td>mouse</td>
<td>5’-TGCAACAGGACCAGAAGTGGA-3’</td>
<td>5’-CACATCAAGCTCCCCACCA-3’</td>
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</tbody>
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Supplementary references


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stained with Alcian blue, in rat colon carcinogenesis induced with 1,2-dimethylhydrazine dihydrochloride. *Cancer Sci* **95**: 792-797