Science Translational Medicine
AAAS

www.sciencetranslationalmedicine.org/cgi/content/full/10/427/eaam7610/DC1

Supplementary Materials for

Targeting p53-dependent stem cell loss for intestinal chemoprotection

Brian J. Leibowitz, Liheng Yang, Liang Wei, Monica E. Buchanan, Madani Rachid, Robert A. Parise, Jan H. Beumer, Julie L. Eiseman, Robert E. Schoen, Lin Zhang, Jian Yu*

*Corresponding author. Email: yuj2@upmc.edu

Published 7 February 2018, *Sci. Transl. Med.* **10**, eaam7610 (2018)
DOI: 10.1126/scitranslmed.aam7610

This PDF file includes:

Materials and Methods
Fig. S1. *PUMA* KO inhibits CPT-11–induced crypt apoptosis.
Fig. S2. CPT-11 causes dose-dependent lethality in mice.
Fig. S3. A small-molecule PUMAi does not protect colon cancer cells against CPT-11–induced apoptosis.
Fig. S4. PUMAi inhibits CPT-11–induced CBC apoptosis.
Fig. S5. PUMAi protects against chemotherapy- and radiation-induced lethality.
Fig. S6. Targeting PUMA does not compromise tumor response to CPT-11.
Fig. S7. 5-FU–induced LGR5^{+} cell apoptosis is PUMA-dependent.
Fig. S8. *Puma* KO and PUMAi suppress CPT-11–induced expression of WNT and NOTCH targets.
Table S1. Treatment-associated lethality in LLC tumor experiments.
Table S2. Mouse-specific primers used for real-time reverse transcription polymerase chain reaction.
Table S3. Human-specific primers used for real-time reverse transcription polymerase chain reaction.
References (*58*, *59*)

Materials and Methods

Mice and treatment

CPT-11 (irinotecan hydrochloride; Camptosar, Pfizer) treatments were administered at 200 mg/kg i.p. unless otherwise indicated. PUMA inhibitor (PUMAi) was prepared as a 50 mg/ml stock in DMSO, then freshly diluted to 2 mg/ml in PBS, and given at 10 mg/kg i.p. 2 hours before chemotherapy treatment unless otherwise indicated. For survival experiments, mice were treated with CPT-11 for three consecutive days. PUMAi was given 4 times, 2 hours before each chemotherapy and 22 hours after the last.

For tumor experiments, mice were treated with CPT-11 three times per week for two weeks. Mice receiving PUMAi were treated 2 hours before each dose of CPT-11 and 20 hours after. Mice were sacrificed four hours after the final CPT-11 treatment (fig. S6A). Tumor volumes were measured with calipers and calculated as volume = (length x width^2)/2. Mice were 7-9 weeks old, and roughly equal numbers of male and female animals were used. To account for variations in starting body weights, the values in Figures 3 and 4 are expressed as a percentage of the respective mouse weights at the time of the first CPT-11 treatment.

5-fluorouracil (5-FU) (APP Pharmaceuticals) treatments were administered at 200 mg/kg i.p. For total body irradiation experiments, PUMAi (10 mg/kg) was given 30 minutes before and 30 minutes after irradiation, followed by daily doses for four days.

IHC and IF

Active caspase 3 IHC. Sections were deparaffinized and rehydrated through graded ethanols. Rehydrated sections were then treated with 3% hydrogen peroxide, followed by antigen retrieval for 10 minutes in boiling 0.1 M citrate buffer (pH 6.0) with 1 mM EDTA. Non-specific antibody binding was blocked using 20% goat serum (Invitrogen) at room temperature for 30 minutes. Sections were incubated overnight at 4°C in a humidified chamber with 1:100 diluted rabbit-anti-caspase-3 (cleaved, Asp 175) (9661; Cell Signaling Technologies). Sections were then incubated for 1 hour at room temperature with biotinylated goat anti-rabbit secondary antibodies (#31822; Pierce) and developed with an ABC kit and DAB (Vector Laboratories).

LGR5 (GFP) IF. Sections were deparaffinized and rehydrated through graded ethanols. Antigen retrieval was performed by boiling for 10 minutes in 0.1 M citrate buffer (pH 6.0) with 1 mM EDTA. Non-specific antibody binding was blocked using 20% goat serum (Invitrogen) at room temperature for 30 min. Sections were incubated overnight at 4°C in a humidified chamber with 1:50 diluted mouse anti-GFP (sc-9996; Santa Cruz Biotechnology). Sections were then incubated with AlexaFluor 488-conjugated goat anti-mouse secondary antibodies (1:200; AA11001; Invitrogen) for 1 hour at room temperature (*58*). Sections were then washed in PBS and mounted with VectaShield + DAPI (Vector Labs).

LGR5(GFP)/TUNEL IF. Sections were prepared as described above. Non-specific antibody binding was blocked using 20% goat serum (Invitrogen) at room temperature for 30 min. Sections were incubated overnight at 4°C in a humidified chamber with 1:50 diluted mouse anti-GFP (sc-9996; Santa Cruz Biotechnology). Sections were then incubated with AlexaFluor 594-

conjugated goat anti-mouse secondary antibodies (1:200; AA11005; Invitrogen) for 1 hour at room temperature. Sections were then washed in PBS, and TUNEL staining was performed with the ApopTag Fluorescein In Situ Apoptosis Detection Kit (Chemicon) according to the manufacturer's instructions (*37*, *46*).

MMP7 IF. Sections were prepared as described above. Non-specific antibody binding was blocked using 20% chicken serum (Invitrogen) at room temperature for 30 min. Sections were washed in PBS and incubated overnight at 4°C in a humidified chamber with 1:100 diluted goat anti-MMP7 (AF2967; R&D Systems). Sections were then incubated with AlexaFluor 594-conjugated rabbit anti-goat secondary antibodies (1:200; AA11080; Invitrogen) for 1 hour at room temperature. Sections were then washed in PBS and mounted with VectaShield + DAPI (Vector Labs) for visualization.

LGR5(GFP)/MMP7 IF. Sections were prepared as described above. Non-specific antibody binding was blocked using 20% chicken serum (Invitrogen) at room temperature for 30 min. Sections were then stained for GFP as described above, followed by staining for MMP7 as described.

PCNA IF. Sections were prepared as described above. Non-specific antibody binding was blocked using 20% goat serum (Invitrogen) at room temperature for 30 min. Sections were incubated overnight at 4°C in a humidified chamber with 1:100 diluted mouse-anti-PCNA (sc-56; Santa Cruz). Sections were then incubated with AlexaFluor 594-conjugated goat anti-mouse

secondary antibodies (1:200; Invitrogen) for 1 hour at room temperature. Sections were then washed in PBS and mounted with VectaShield + DAPI (Vector Labs) (*18*).

CD166 IF. Sections were prepared as described above. Non-specific antibody binding was blocked using 20% rabbit serum (Pierce) at room temperature for 30 min. Sections were incubated overnight at 4°C in a humidified chamber with goat anti-mouse CD166 antibodies (1:100; AF1172; R&D Systems). Sections were then incubated with AlexaFluor-594 rabbit anti-goat secondary antibodies (1:200; A11080; Invitrogen) for 1 hour at room temperature and counterstained with VectaShield plus DAPI (*59*).

p53 BP1 IF: Sections were prepared as described above. Non-specific antibody binding was blocked using 20% goat serum (Invitrogen) at room temperature for 30 min. Sections were incubated overnight at 4°C in a humidified chamber with rabbit anti-mouse 53BP1 antibodies (1:100; IHC-00001; Bethyl Laboratories) (*46*). Sections were then incubated with AlexaFluor-594 goat anti-rabbit secondary antibodies (1:200; A11012; Invitrogen) for 1 hour at room temperature and counterstained with VectaShield plus DAPI.

Neutrophil IF. Sections were prepared as described above. Non-specific antibody binding was blocked using 20% goat serum (Invitrogen) at room temperature for 30 min. Sections were incubated overnight at 4°C in a humidified chamber with rat anti-mouse Ly-6B.2 (1:100; MCA771GT; AbD Serotec) (*56*). Sections were then incubated with AlexaFluor-594 goat anti-rat secondary antibodies (1:200; A11007; Invitrogen) for 1 hour at room temperature and counterstained with VectaShield plus DAPI.

LC-MS/MS quantitation of PUMAi

Acetonitrile and water (all HPLC grade) were purchased from Fisher Scientific. Formic acid and trifluoroacetic acid was purchased from Sigma–Aldrich. Stock solutions of PUMAi and [D_5]-PUMAi internal standard were prepared at 1 mg/mL in DMSO, diluted 10 times in acetonitrile, and then stored at −80 °C. On the day of the assay, the solution was serially diluted (in 10-fold steps) in acetonitrile to obtain the lower calibration working solutions of 0.01 and 0.001 mg/mL. These calibration working solutions were diluted in mouse plasma (Lampire Biological Laboratories, Inc.) to produce the following analyte concentrations: 10, 30, 50, 100, 300, 500, 1000, 3000 ng/mL. For each calibration series, zero (containing 10 ng/mL of internal standard) and blank samples were also prepared from 50 µL of Lampire plasma. Quality control (QC) stock solutions were diluted in Lampire mouse plasma to produce the following QC samples: QC Low (QCL) 20 ng/mL; QC Mid (QCM) 200 ng/mL, and QC High (QCH) 2500 ng/mL.

To extract PUMAi, we added 10 µL of internal standard solution (1000 ng/mL [D_5]-PUMAi in acetonitrile) to 50 µL of plasma sample in a micro centrifuge tube. 250 µL of acetonitrile was added, and the samples were vortexed for 30 s. The sample was centrifuged at 12,000 x g for five min, and the resultant supernatant was evaporated to dryness under nitrogen at 34 °C in 12 x 75 mm glass tubes. The dry residue was reconstituted with 100 µL of acetonitrile/water (v/v), and 10 µL was injected into the LC-MS/MS system.

The LC-MS/MS system consisted of an Agilent 1100 autosampler and binary pump, and a Waters Quattromicro tandem mass spectrometer. The liquid chromatography was performed with a gradient mobile phase consisting of A: acetonitrile/0.1% formic acid (v/v) and B:

water/0.1% formic acid (v/v). The mobile phase was pumped at a flow rate of 0.3 mL/min, and separation was achieved using a Phenomenex Luna 3µm PFP (2) (100a 150x2 mm) column. The gradient mobile phase was as follows: from 0 to 5 min, solvent A was 15%. Solvent A was then increased linearly to 80% at 12 min and maintained at 80% until 16 min. At 16.1 min, solvent A was decreased to 15% and maintained at 15% until 22 min. The run time was 22 min.

The mass spectrometer was operated in ESI positive mode using MRM detection. The parameters of the mass spectrometer were as follows; capillary 1.0 kV, cone voltage 40V, desolvation temperature 400 ℃, desolvation gas flow 550 L/h, cone gas flow 50 L/h, LM and HM resolution 12, collision energy 25 V, and gas cell pirani pressure $1.5*10^{-3}$ mbar. The mass transitions monitored were *m/z* 331>143 for PUMAi and 345>157 for the internal standard.

To assess recovery from plasma, we compared areas from control plasma spiked with 1000 ng/mL of PUMAi, prepared as described above, with the areas obtained with a neat solution of PUMAi in water. Intestinal mucosa was analyzed after dilution in control plasma to within the calibration range. Intestinal mucosa was collected by scraping from a 10 cm portion of the jejunum, and stored at -80C until use. Three mice were used for each treatment group.

Mouse and human colonic organoids

Preparation of WRN-conditioned medium

L-WRN cells (ATCC® CRL-3276) were cultured in DMEM (Invitrogen) supplemented with 1× penicillin/streptomycin (Invitrogen), 0.5 mg/mL G418 (ant-gn-1, InvivoGen), 0.5 mg/mL hygromycin B (10687010, Invitrogen), and 10% FBS (vol/vol) until confluent. Cells were then collected with Trypsin-EDTA (Invitrogen) and split into three T75 cell culture flasks (Sarstedt)

in DMEM supplemented with 1× penicillin/streptomycin and 10% FBS (HyClone) (vol/vol) until they became over-confluent. Cells were then rinsed with 5 ml of primary culture medium (Advanced DMEM/F12 (Invitrogen) supplemented with 1× penicillin/streptomycin, 2 mM GlutaMAX (ThermoFisher), 20% FBS [vol/vol]) and cultured in the same medium (15 ml/flask). Every 24 hours, the primary culture medium (conditioned medium) was collected, and fresh medium was added to the flasks. The conditioned medium was centrifuged at 2,000 *g* for 5 min at room temperature, and the supernatant was decanted and stored in aliquots at -20 ℃ until use.

Mouse crypt isolation, organoids, and treatment

Mouse crypt isolation, organoid development, and passage were done as previously described (*37*) with minor modifications. In brief, the complete culture medium for mouse intestinal organoids was slightly modified as follows: advanced Dulbecco's modified Eagle medium (DMEM)/F12 (12634-010, Invitrogen) was supplemented with 100 units ml^{-1} penicillin/0.1 mg ml^{-1} streptomycin (Invitrogen), 2 mM GlutaMAX (ThermoFisher), 20% (vol/vol) FBS (S11150, ATLANTA Biologicals), and WRN-conditioned medium (50%, vol/vol). Freshly isolated crypts were cultured with the same medium plus 10 μM Y-27632 (Y0503 Sigma) and 100 μg/ml Primocin (ant-pm-1, InvivoGen). For CPT and PUMAi treatment, intestinal organoids were passaged and re-plated in 25 μL of Matrigel (356231, Corning) in 24-well plates 24 hours before treatment with CPT (0.5 μM, C9911, Sigma-Aldrich) and PUMAi (50 μM) for 24 hours. The CPT-containing medium was replaced with complete medium containing PUMAi (50 μM). Three groups (treatment control, CPT only, and CPT plus PUMAi) were set up. Organoids 100 μm or greater in diameter were enumerated on day 6 after CPT treatment. Similar results were obtained from at least three independent experiments with triplicate wells in each experiment.

Human crypt isolation, organoid development, and treatment

Surgically resected intestinal tissues were obtained from Health Science Tissue Bank (HSTB) of UPMC Shadyside Hospital and UPMC Presbyterian Hospital. All samples were obtained with informed consent and approval by the University of Pittsburgh Ethics Committee. Human normal colon crypt isolation and organoid development were performed as previously described (*57*) with modifications. The complete culture medium for normal human colon organoids contains advanced DMEM/F12 (12634-010, Invitrogen) supplemented with 1× penicillin/streptomycin (15140-122, Invitrogen), 10 mM HEPES (15630-106, Invitrogen), 2 mM GlutaMAX (Invitrogen), 1×B27 (17504-044, Invitrogen), 1×N2 (17502-048, Invitrogen), 1 mM *N*-Acetylcysteine (A0737, Sigma), 10 nM [leu-15]-Gastrin (G9145, Sigma), 10 mM nicotinamide (N0636, Sigma), 10 µM SB202190 (S7067, Sigma), 50 ng/ml recombinant murine EGF (315-09, Peprotech), 0.5 µM A83-01 (2939, Tocris Bioscience), 10 nM PGE2 (22-961-0, Tocris Bioscience), and 50% WRN-conditioned medium (vol/vol). Freshly isolated crypts were cultured with the same medium plus 10 µM Y-27632 and 100 µg/ml Primocin. Organoids 100 µm or greater in diameter were enumerated on day 6 after CPT treatment. Similar results were obtained from at least three independent experiments with triplicate wells in each experiment.

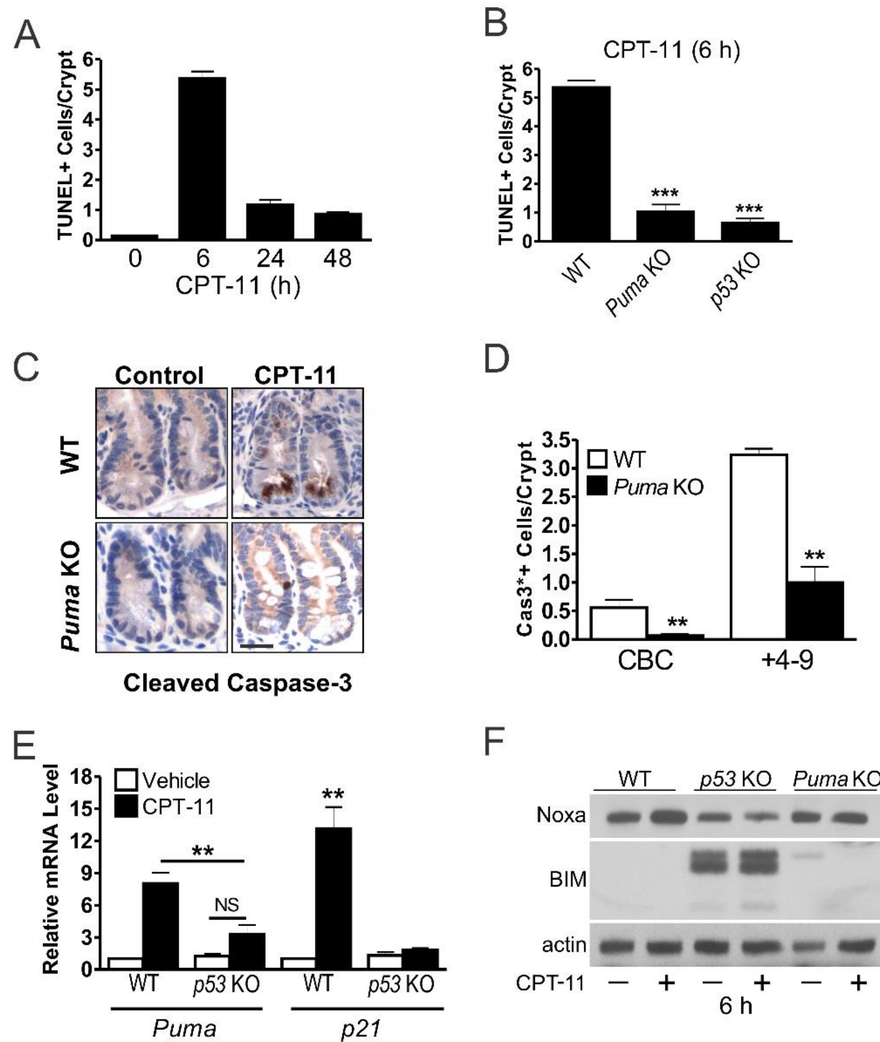

Fig. S1. *PUMA* KO inhibits CPT-11–induced crypt apoptosis. Mice were treated with a single dose of CPT-11 (200 mg/kg), and analyzed at indicated times. (**A**) Quantitation of TUNEL$^+$ crypt cells in WT mice at indicated times. (**B**) Quantitation of TUNEL$^+$ crypt cells at 6 h. (**C**) Representative images of active caspase-3 staining in the crypts at 6 h. Bar = 25 µm. (**D**) Quantitation of active caspase-3$^+$ CBC and +4-9 cells at 6 h. (**E**) Indicated mRNAs were analyzed by qRT-PCR. cDNAs were synthesized from RNA pooled from 3 mice. **(F)** Indicated proteins at 6 h were analyzed by western blotting. Lysates were pooled from intestinal mucosa

from 3 mice. Representative results are shown, and similar results were obtained in at least three independent experiments. A, B, D, E, values are mean ± SEM, n = 3 mice per group. **P < 0.01; ***P < 0.001 (Student’s T Test, two-tailed).

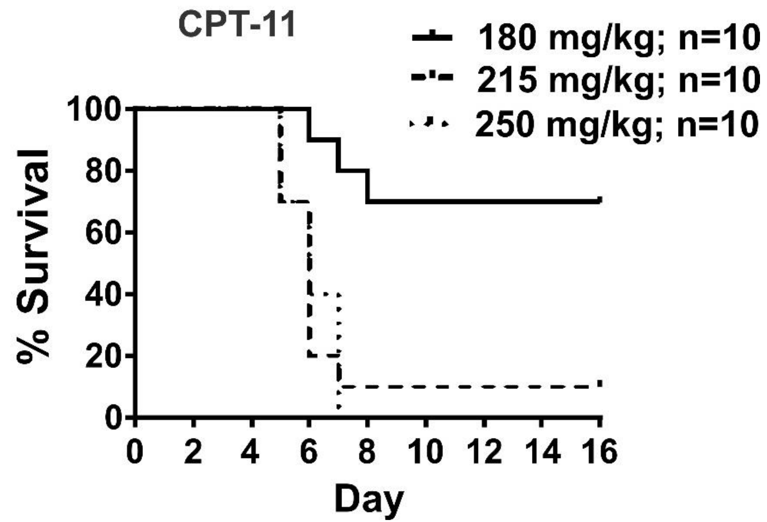

Fig. S2. CPT-11 causes dose-dependent lethality in mice. Survival of WT mice treated with three consecutive daily doses of 180, 215, or 250 mg/kg/day CPT-11 on days 0, 1, and 2. n = 10 mice per group. P values were calculated by log-rank test.

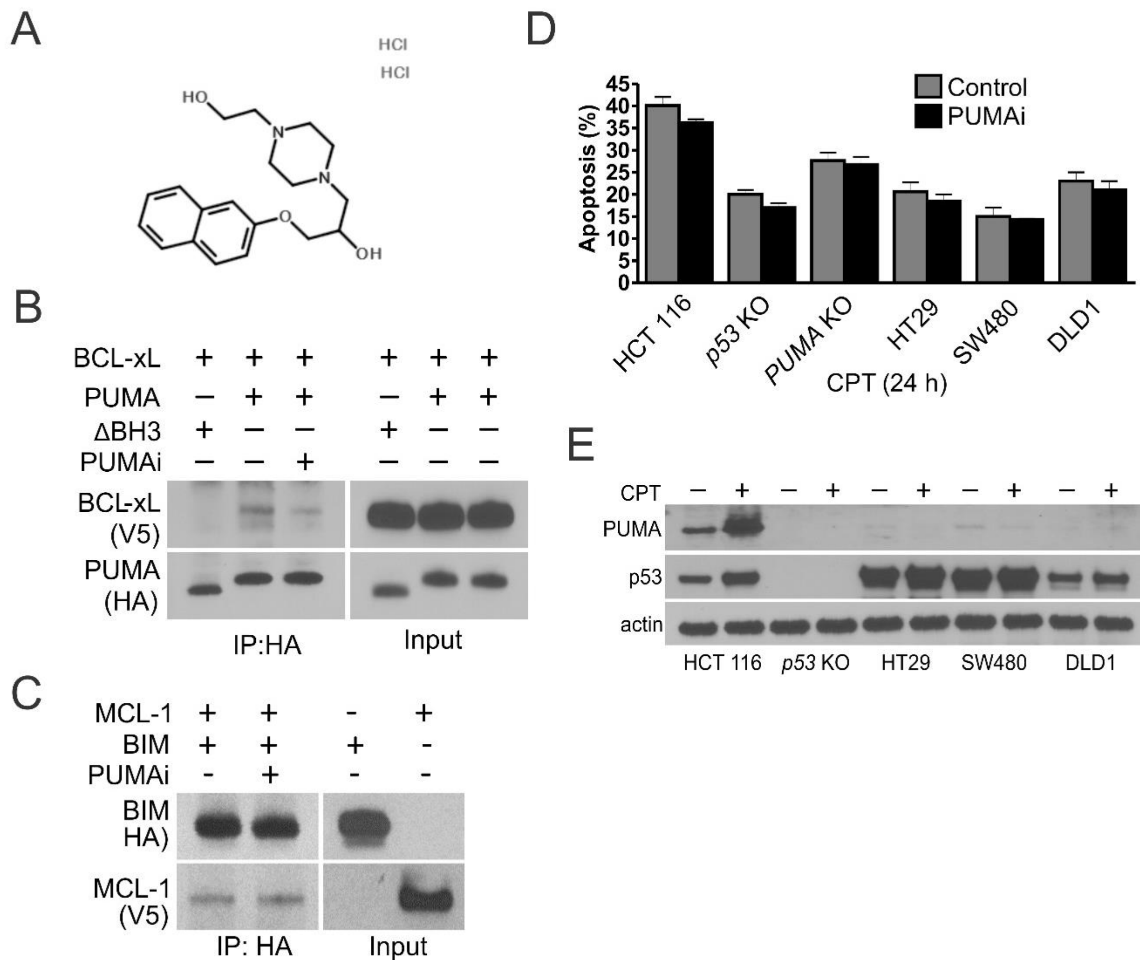

Fig. S3. A small-molecule PUMAi does not protect colon cancer cells against CPT-11–induced apoptosis. (**A**) Structure of the PUMA inhibitor lead compound (PUMAi). (**B**) Lysates of 293 cells transfected with individual plasmids for 24 hours were mixed in the indicated combinations. Lysates were incubated with vehicle or PUMA inhibitor (PUMAi, 25 μM) for 15 min before immunoprecipitation with an anti-HA antibody. The HA-bound proteins and input were analyzed by western blotting. (**C**) Lysates of 293 cells transfected with individual plasmids for 24 h were mixed in the indicated combinations. Lysates were incubated with vehicle or PUMA Inhibitor (PUMAi, 25 μM) for 15 min before immunoprecipitation with an anti-HA

antibody. The HA-bound proteins and input were analyzed by western blotting. (**D)** Indicated colon cancer cell lines were treated with camptothecin (CPT, 500 nM) for 24 h. HCT 116 cells and *PUMA* KO HCT 116 cells have WT p53, others have no (KO) or mutant p53. Apoptosis was measured by nuclear fragmentation assay. Values are mean + SEM; n = 3 independent experiments. (**E**) Cells were treated as in D for 24 h and analyzed by western blotting. Representative results are shown, and similar results were obtained in at least three independent experiments.

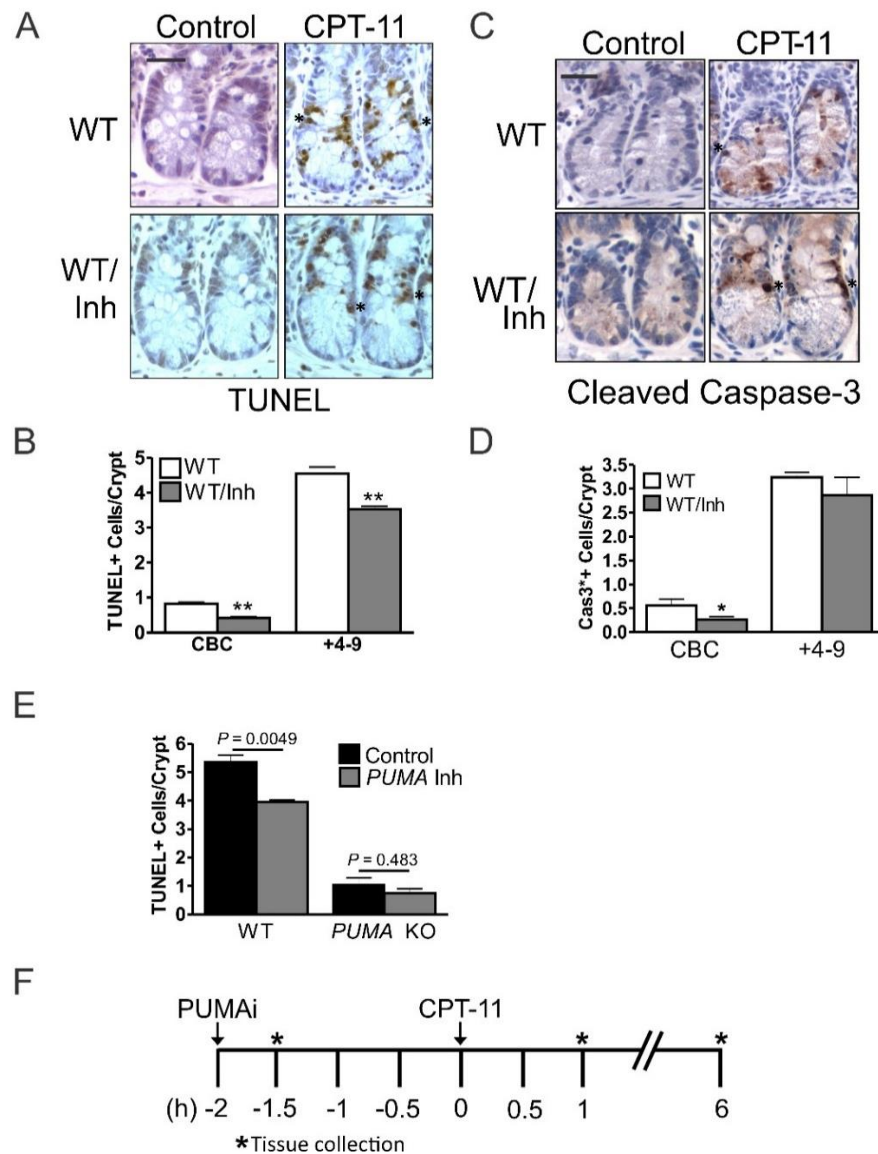

Fig. S4. PUMAi inhibits CPT-11–induced CBC apoptosis. Mice were treated with a single dose of CPT-11 (200 mg/kg), and analyzed at 6 h. PUMAi was given 2 h before CPT-11. (**A**) Representative images of TUNEL staining in intestinal crypts of WT mice with indicated treatment. Bar = 25 µm. (**B**) Quantitation of TUNEL$^+$ crypt cells at positions 1-3 (CBC) or 4-9. (**C**) Representative images of active caspase-3 staining in intestinal crypts. Bar = 25 µm. (**D**) Quantitation of active caspase-3$^+$ crypt cells at positions 1-3 (CBC) or 4-9. (**E**) Quantitation of

TUNEL$^+$ crypt cells. **(F)** Schematic of treatment and tissue collection schedule for PUMAi pharmacokinetic experiment. CPT -11 was administered at 0 h. B, D, and E, values are mean ± SEM, n = 3 mice per group. A and C, asterisks indicate TA cells. **P < 0.01. *P < 0.05 (Student's T Test, two-tailed).

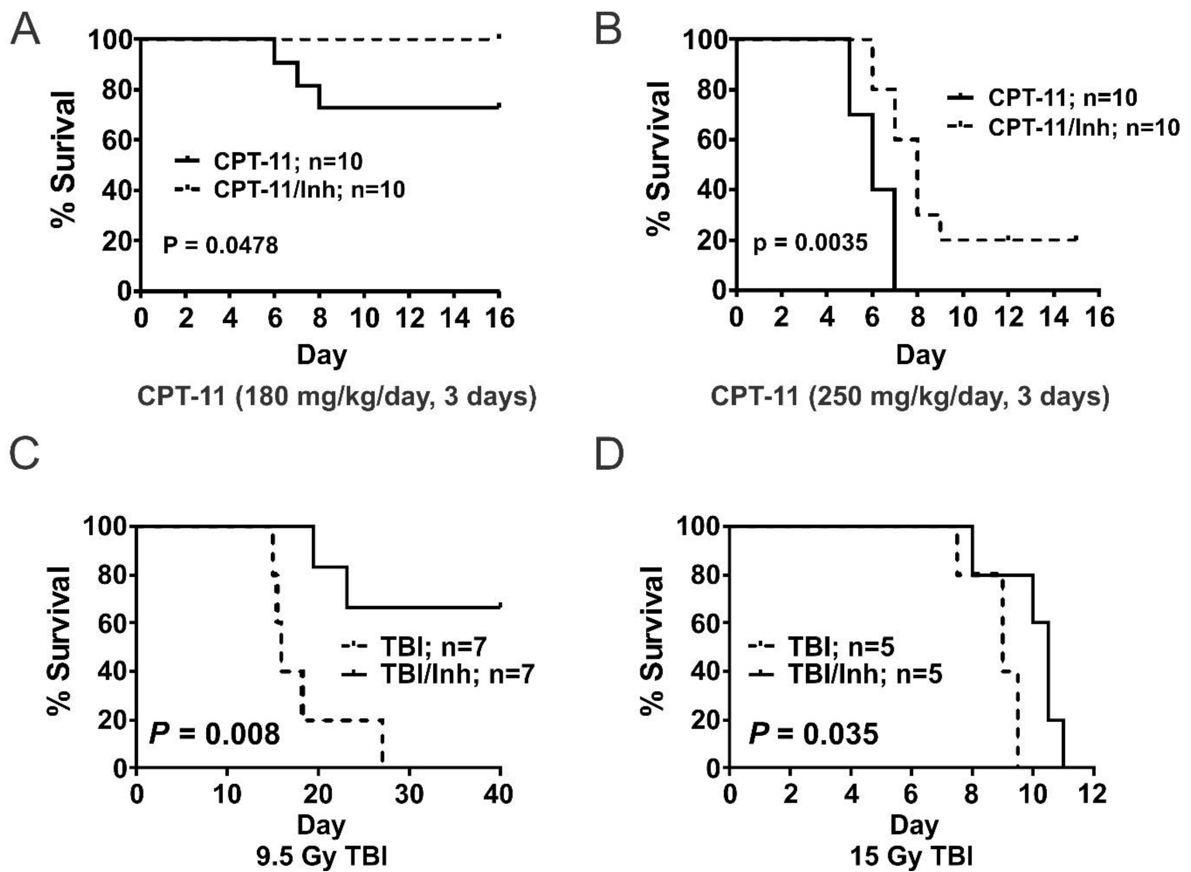

Fig. S5. PUMAi protects against chemotherapy- and radiation-induced lethality. (A) and **(B)** Survival of WT mice treated with 3 daily doses of 180 mg/kg/day (A) and 250 mg/kg/day (B) CPT-11, respectively, on days 0, 1, and 2. PUMAi (10 mg/kg) was given 2 hours before each dose of CPT-11 and 20 hours after the final dose. **(C)** and **(D)** Survival of WT mice after total body irradiation (TBI) at 9.5 Gy (C) and 15 Gy (D). PUMAi (10 mg/kg) was given 30 min before and 30 min after TBI, and then daily for 4 days. Survivors at 9.5 Gy TBI in **C** were sacrificed on day 40 with no obvious health problems. P values were calculated by log-rank test.

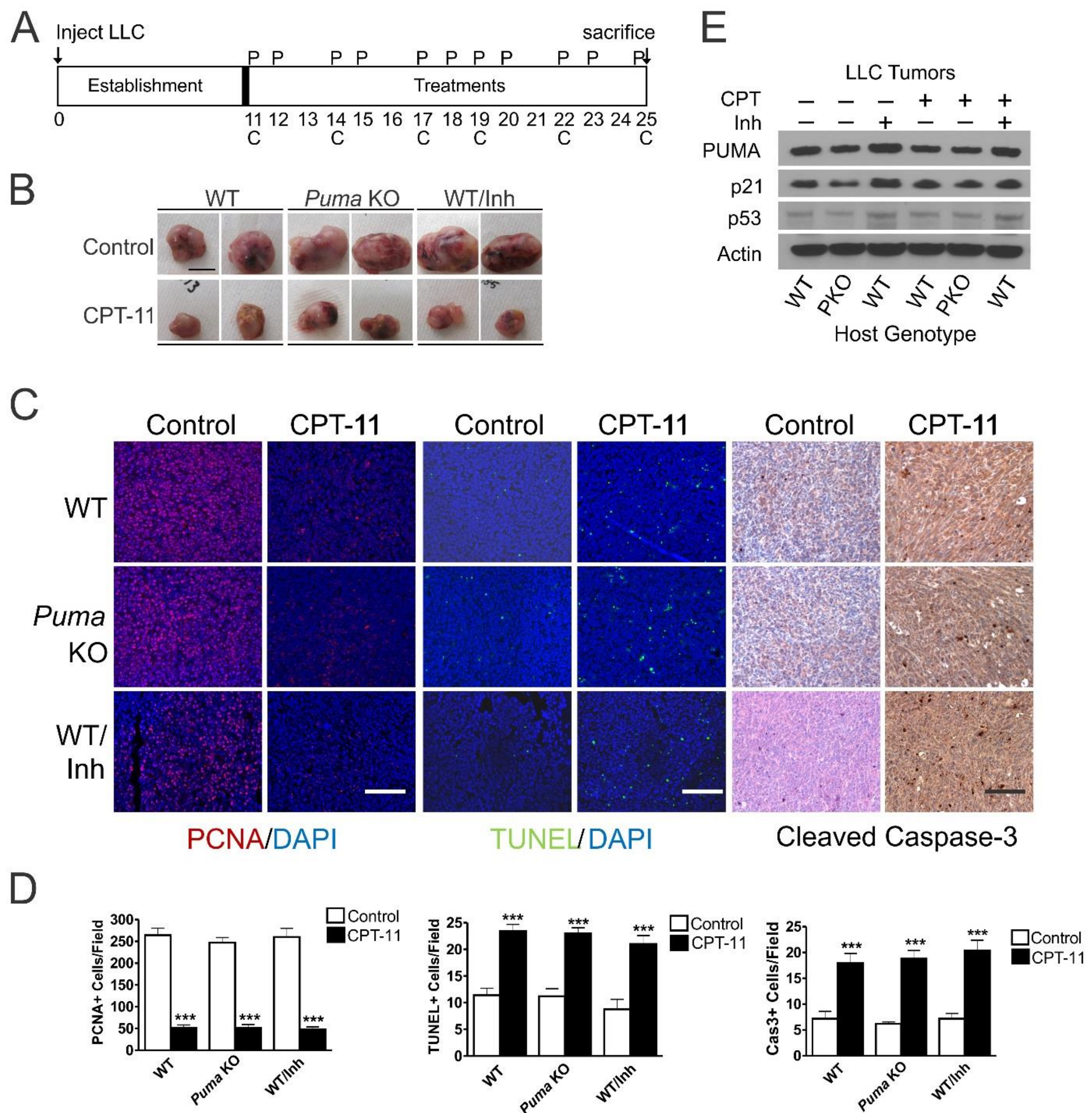

Fig. S6. Targeting PUMA does not compromise tumor response to CPT-11. (**A**) Schematic for tumor establishment and treatments. Mice were injected subcutaneously on their flanks with four million Lewis Lung Carcinoma (LLC) cells. Tumors were allowed to establish for 11 days, and mice were treated with 200 mg/kg CPT-11 (C) 6 times over 2 weeks. PUMAi (P) was given 11 times, 2 hours before and 20 hours after each dose of CPT-11. Mice were sacrificed 4 hours

after the last dose of CPT-11. (**B**) Representative images of tumors collected after two weeks of treatment. Bar = 1 cm. (**C**) Representative images of PCNA, TUNEL, and active (cleaved) caspase-3 staining in LLC tumors after two weeks of CPT-11 treatment. Bar = 100 µm. (**D**) Quantitation of $PCNA^+$, $TUNEL^+$, and cleaved caspase-3^+ cells per field in C. Values are mean ± SEM, n = 3 tumors per group. **P < 0.01. ***P < 0.001 (Student's T Test, two-tailed). (**E**) Western blots of the indicated proteins from tumors treated as in **A**. Each lane represents a single tumor.

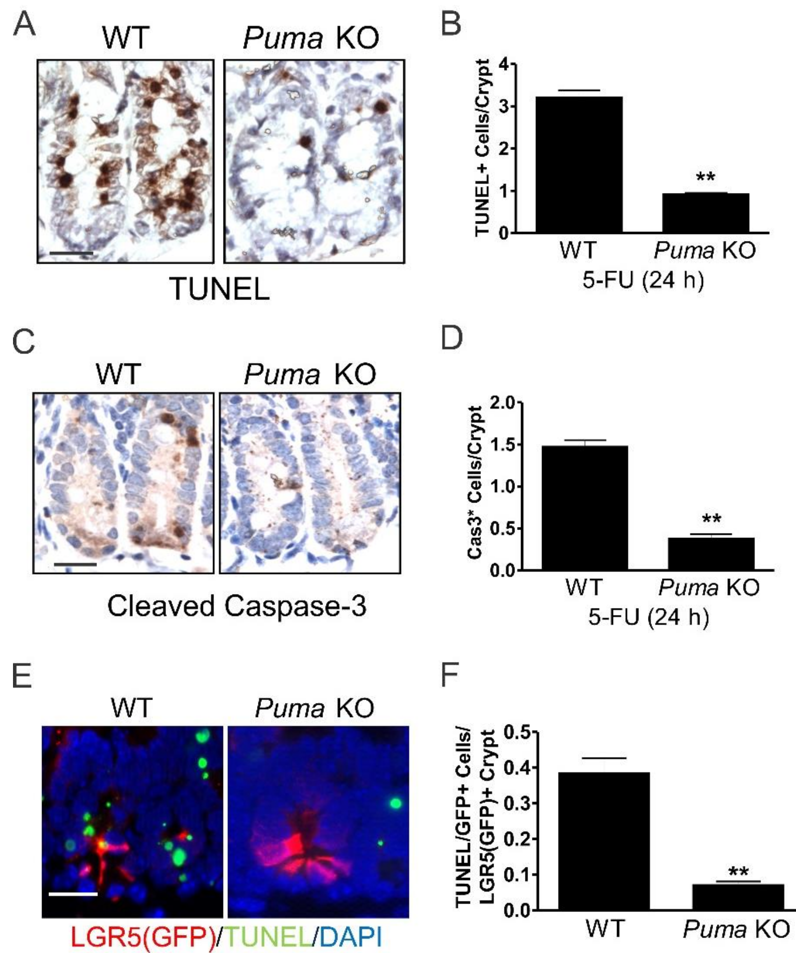

Fig. S7. 5-FU–induced LGR5⁺ cell apoptosis is PUMA-dependent. WT and *Puma* KO LGR5-EGFP mice were treated with 200 mg/kg 5-FU for 24 h. (**A**) Representative images of TUNEL staining in intestinal crypts. Bar = 25 µm. (**B**) Quantitation of $TUNEL^+$ crypt cells. (**C**) Representative images of cleaved caspase-3 staining in intestinal crypts. Bar = 25 µm. (**D**) Quantitation of active (cleaved) caspase-3^+ crypt cells. (**E**) Representative images of LGR5 (GFP)/TUNEL double immunofluorescence in intestinal crypts. (**F**) Quantitation of GFP/TUNEL double positive cells in GFP-positive crypts. B, D, and F, values are mean ± SEM, n = 3 mice per group. **P < 0.01 (Student's T Test, two-tailed).

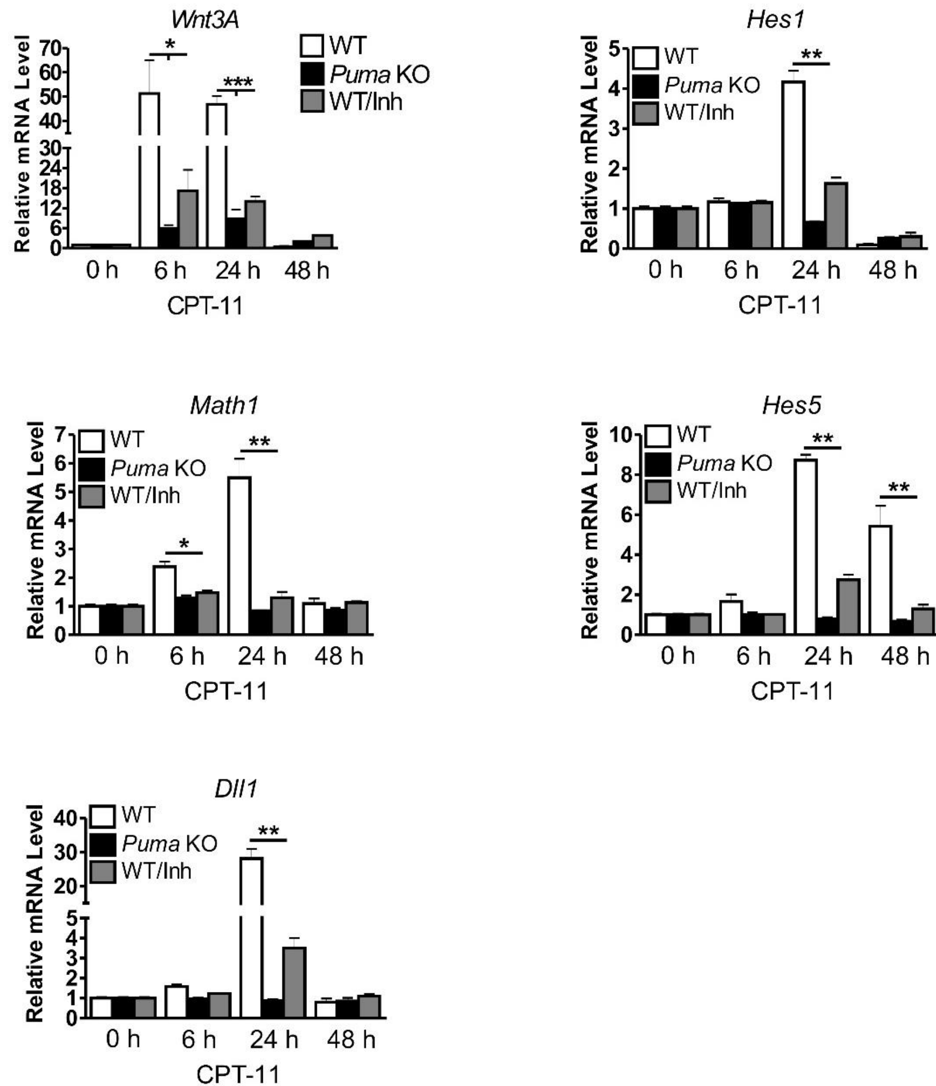

Fig. S8. ***Puma*** **KO and PUMAi suppress CPT-11–induced expression of WNT and NOTCH targets.** Indicated mRNAs were analyzed by qRT-PCR. cDNAs were synthesized from RNA pooled from 3 mice. Values for each gene were normalized to *Gapdh*, and expressed relative to their own 0 h controls. $^{*}P < 0.05$, $^{**}P < 0.01$ (one-way ANOVA with TUKEY post-hoc test performed separately for each time point).

Table S1. Treatment-associated lethality in LLC tumor experiments.

Genotype	Treatment Group	% mice completing treatment
WT	Vehicle Control	100% (8/8)
WT	CPT-11	73% (8/11)
PUMA KO	Vehicle Control	100% (6/6)
PUMA KO	CPT-11	100% (6/6)
WT	PUMAi	100% (5/5)
WT	CPT-11 + PUMAi	100% (10/10)

Table S2. Mouse-specific primers used for real-time reverse transcription polymerase chain reaction.

Gene	Primer	Sequence
Puma	Forward	5´-ATGGCGGACGACCTCAAC-3´
	Reverse	5´-AGTCCCATGAAGAGATTGTACATGAC-3´
p21	Forward	5´-ATGTCCAATCCTGGTGATGT-3´
	Reverse	5´-TGCAGCAGGGCAGAGGAAGT-3´
Lgr5	Forward	5´-GACAATGCTCTCACAGAC-3´
	Reverse	5´-GGAGTGGATTCTATTATTATGG-3´
Wnt3A	Forward	5' CACCACCGTCAGCAACAGCC 3'
	Reverse	5' AGGAGCGTGTCACTGCGAAAG 3'
CD44	Forward	5′-ACTTTCTGCCCCTCTCCACT-3′
	Reverse	5' AGGAGCGTGTCACTGCGAAAG 3'
Olfm4	Forward	5´-GCCACTTTCCAATTTCAC-3´
	Reverse	5´-GAGCCTCTTCTCATACAC-3´
Math1	Forward	5'-ACATCTCCCAGATCCCACAG-3'
	Reverse	5'-GGGCATTTGGTTGTCTCAGT-3'
Hes1	Forward	5'-CTCCCGGCATTCCAAGCTAG-3'
	Reverse	5'-AGCGGGTCACCTCGTTCATG-3'
Hes5	Forward	5′-AGTCCCCAAGGAGAAAAACCGA-3′
	Reverse	5′-GCTGTGTTTCAGGTAGCTGAC-3′
Dll1	Forward	5′-TGAGCCAGTCTTTCCTTGAA-3′
	Reverse	5′-AGACCCGAAGTGCCTTTGTA-3′
Gapdh	Forward	5´-CTCTGGAAAGCTGTGGCGTGATG-3´
	Reverse	5´-ATGCCAGTGAGCTTCCCGTTCAG-3´

Table S3. Human-specific primers used for real-time reverse transcription polymerase chain reaction.

Gene	Primer	Sequence
LGR5	Forward	5’-AACAGTCCTGTGACTCAACTCAAG-3’
	Reverse	5’-TTAGAGACATGGGACAAATGCCAC-3’
OLFM4	Forward	5'-CTGCCAGACACCACCTTTCC-3'
	Reverse	5'-CTCGAAGTCCAGTTCAGTGTAAG-3'
CD44	Forward	5’-GACAAGTTTTGGTGGCACG-3’
	Reverse	5’-CACGTGGAATACACCTGCAA-3’
ACTIN	Forward	5´-GACCTCACAGACTACCTCAT -3´
	Reverse	5´-AGACAGCACTGTGTTGGCTA-3´