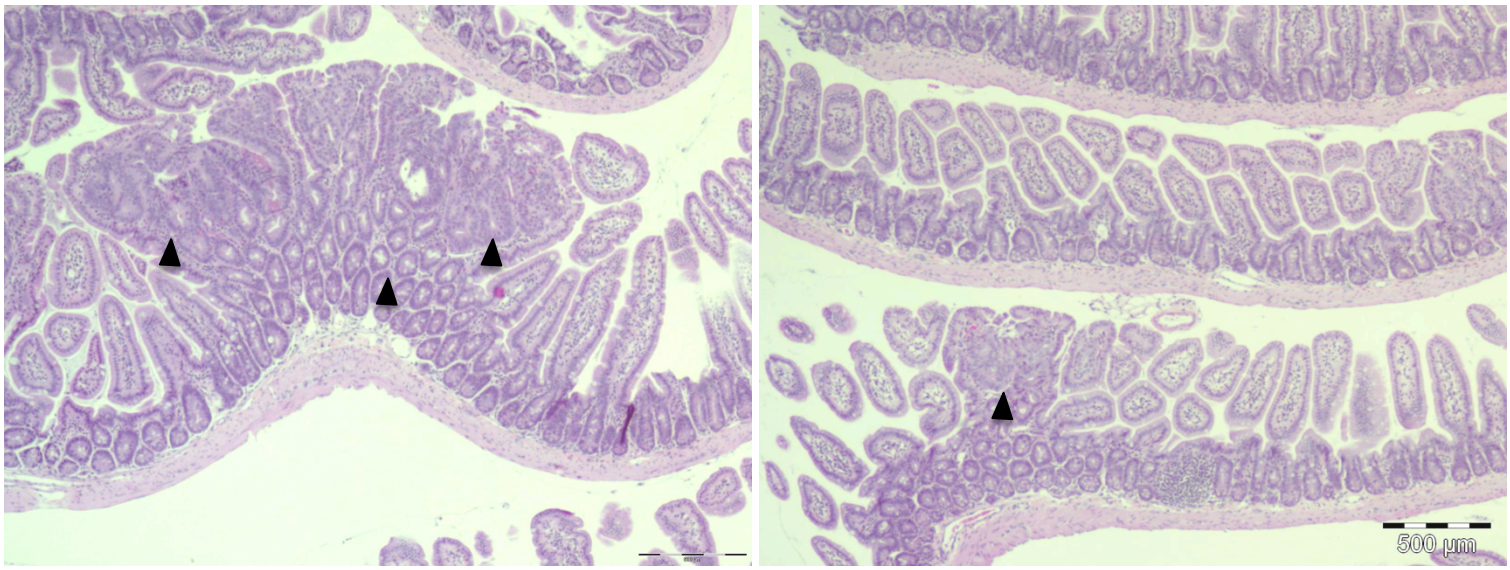
Prevention and treatment of colon cancer by peroral administration of HAMLET (human α-lactalbumin made lethal to tumor cells)

Manoj Puthia, Petter Storm, Aftab Nadeem, Sabrina Hsiung, Catharina Svanborg

Supplementary Figure 1



Control HAMLET treated

Supplementary Figure 1. Histology of H&E stained intestinal sections from HAMLET treated and control $APC^{Min/+}$ mice. Intestinal villus hyperplasia was noticed in sham-treated controls but not in HAMLET-treated mice (arrowhead, tumor; scale bars, 500 µm).

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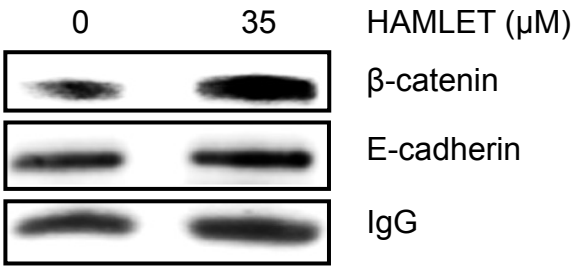
Supplementary Figure 2



Supplementary Figure 2. HAMLET enhances β-catenin and E-cadherin binding in colon cancer cells DLD1. E-cadherin was pulled down in immunoprecipitation assay and Western blot was performed for various proteins. Although, E-cadherin levels remained unchanged, an increase in E-cadherin bound β-catenin was observed after HAMLET treatment (3 h). This confirms enhanced colocalization of β-catenin with E-cadherin on cell membrane (see Fig. 3F). IgG shows equal loading.

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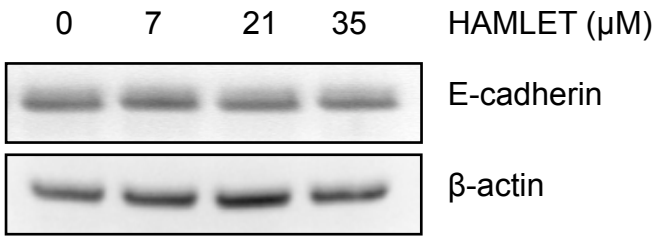
Supplementary Figure 3



Supplementary Figure 3. HAMLET does not change E-cadherin expression in colon cancer cells DLD1. Western blot analysis of whole cell extracts demonstrated no change of E-cadherin protein after HAMLET treatment. β-actin was used as the loading control.

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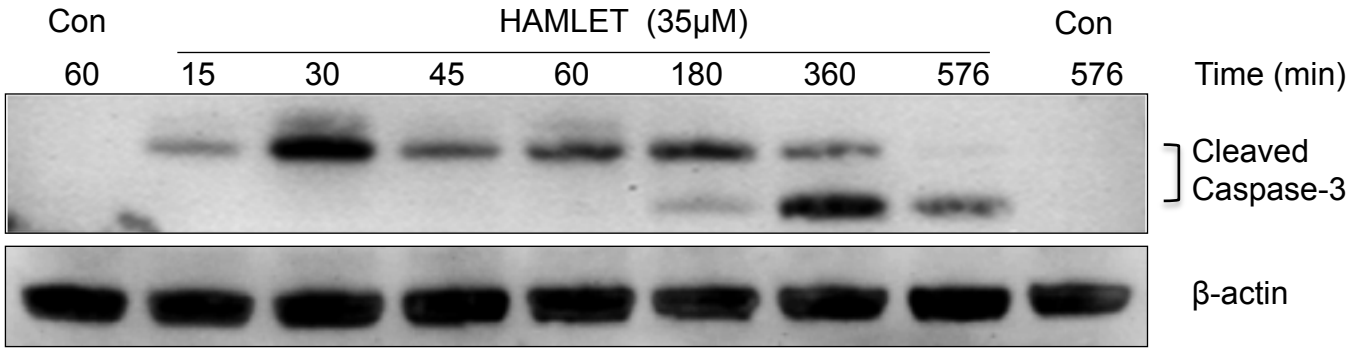
Supplementary Figure 4



Supplementary Figure 4. HAMLET activates caspase-3 in colon cancer cells DLD1. Immunoblot demonstrating activation of caspase-3 in DLD1 cells in response to HAMLET treatment. Caspase-3 activation was observed as early as 15 minute after HAMLET treatment. Two bands (19 and 17 kDa) of cleaved caspase-3 can be seen on the blot. β-actin was used as the loading control.

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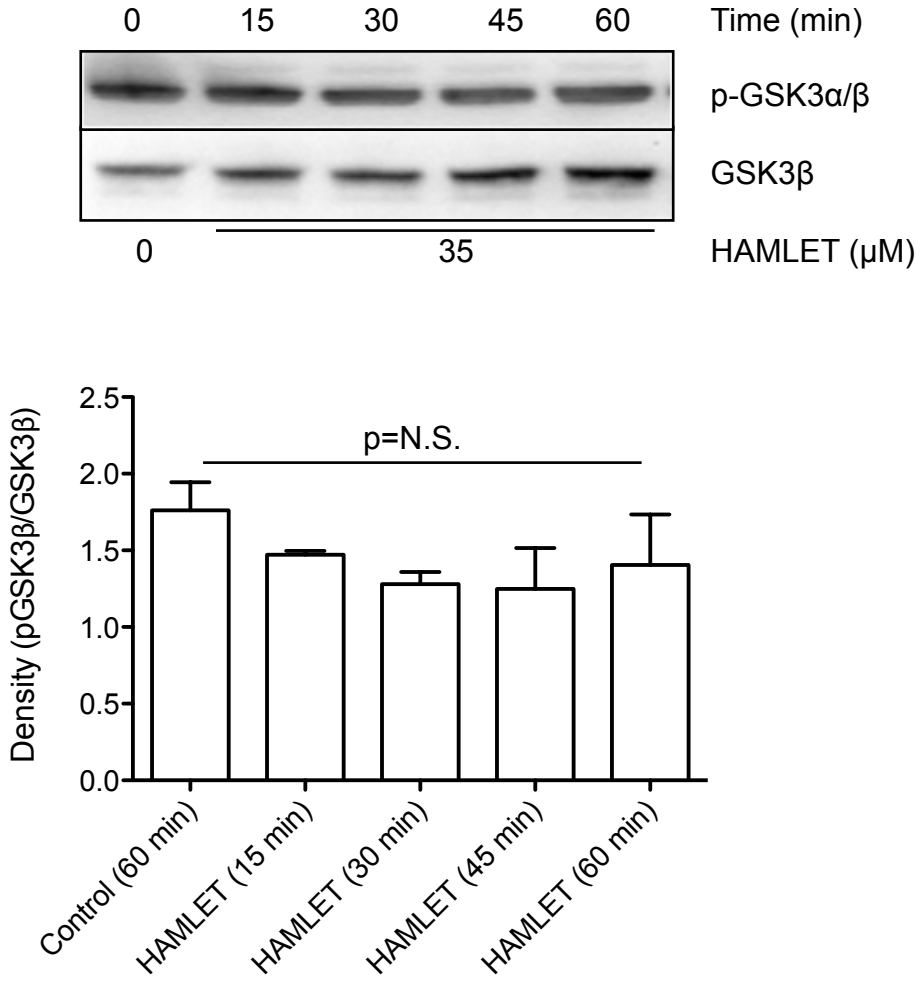
Supplementary Figure 5



Supplementary Figure 5. HAMLET does not change p-GSK3α/β levels in colon cancer cells DLD1. Western blot analysis of whole cell extracts demonstrated that p-GSK3α/β protein levels do not change after HAMLET treatment. Total GSK3β protein levels were used as control. Histogram shows average density of bands (*n* = 2). N.S., not significant.

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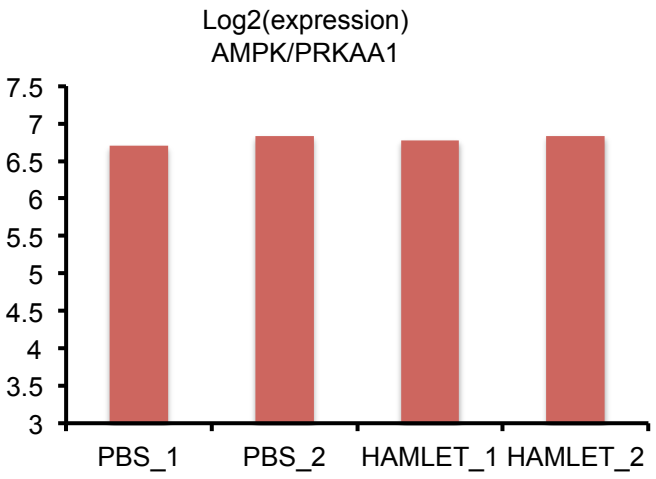
Supplementary Figure 6



Supplementary Figure 6. AMPK RNA levels in DLD-1 cells treated with HAMLET. DLD-1 cells were seeded overnight and treated with HAMLET (35 μM) for three hours. Global expression profiles were determined using Affymetrix HGU219 arrays. HAMLET did not alter the expression of AMPK *in vitro*. Normalized expression values are shown for AMPK (PRKAA1) in duplicate of PBS- and HAMLET-treated DLD1-cells.

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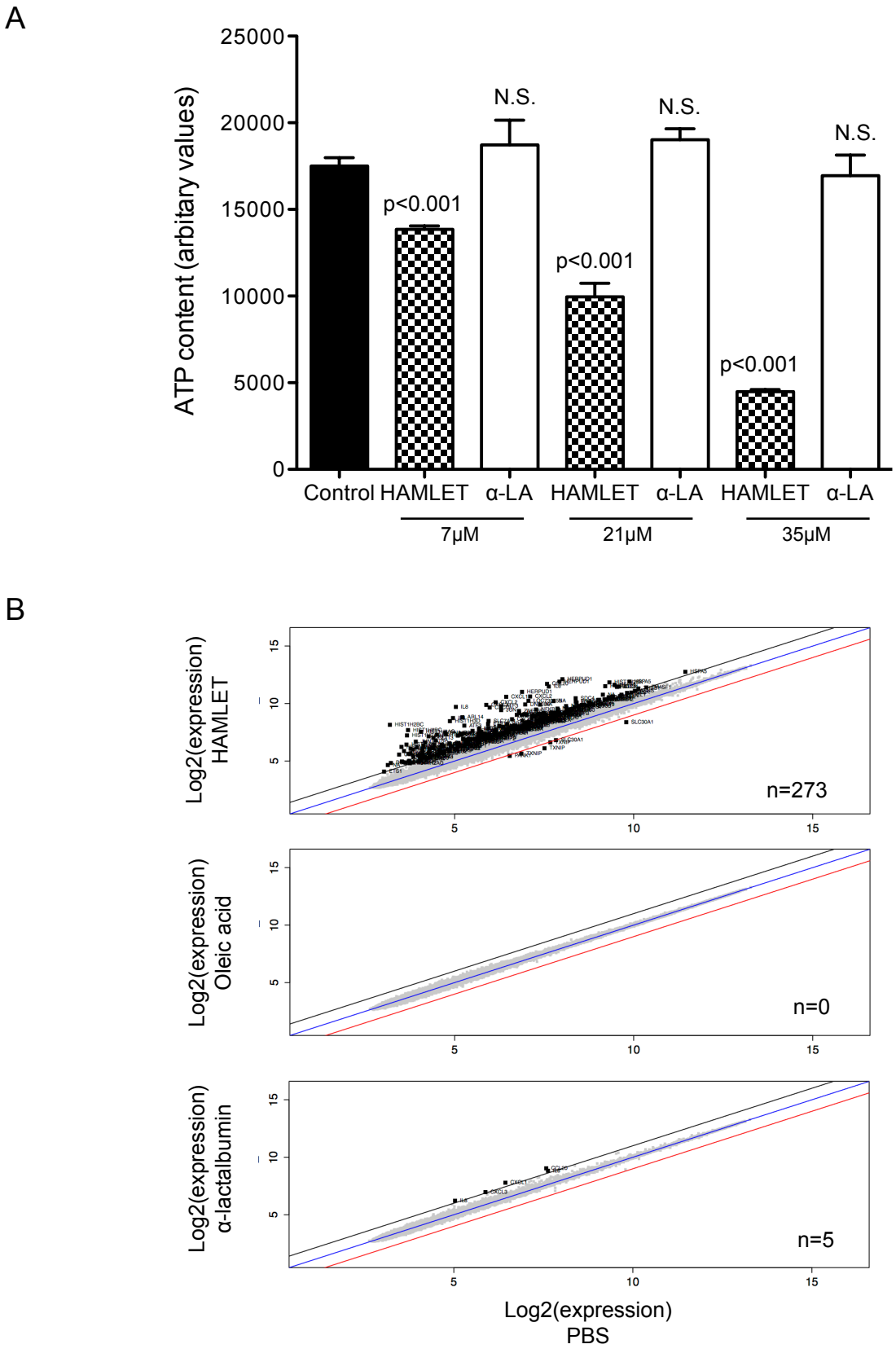
Supplementary Figure 7



Supplementary Figure 7. Effects of α-lactalbumin and oleic acid on gene expression and cell viability. (**A**) α-lactalbumin alone showed no effect on ATP levels in DLD-1 cells. *P* values, compared to control. *n* = 2; α-LA, α-lactalbumin. (**B**) Expression profiling of DLD-1 cells treated with HAMLET, oleic acid or α-lactalbumin. DLD-1 cells were seeded overnight and treated with HAMLET (35 μM), oleic acid (175 μM) or a-lactalbumin (35 μM) for three hours. Global expression profiles were determined using Affymetrix HGU219 arrays. Scatter plots of RMA-normalized expression for each genes with HAMLET, oleic acid or α-lactalbumin on the y-axis and PBS-control on the x-axis. HAMLET induced differential expression of 273 genes, whereas oleic acid induced no differentially expressed genes and α-lactalbumin induced only 5 at a cutoff of log2-foldchange > 1 and FDR-adjusted p-value < 0.05.

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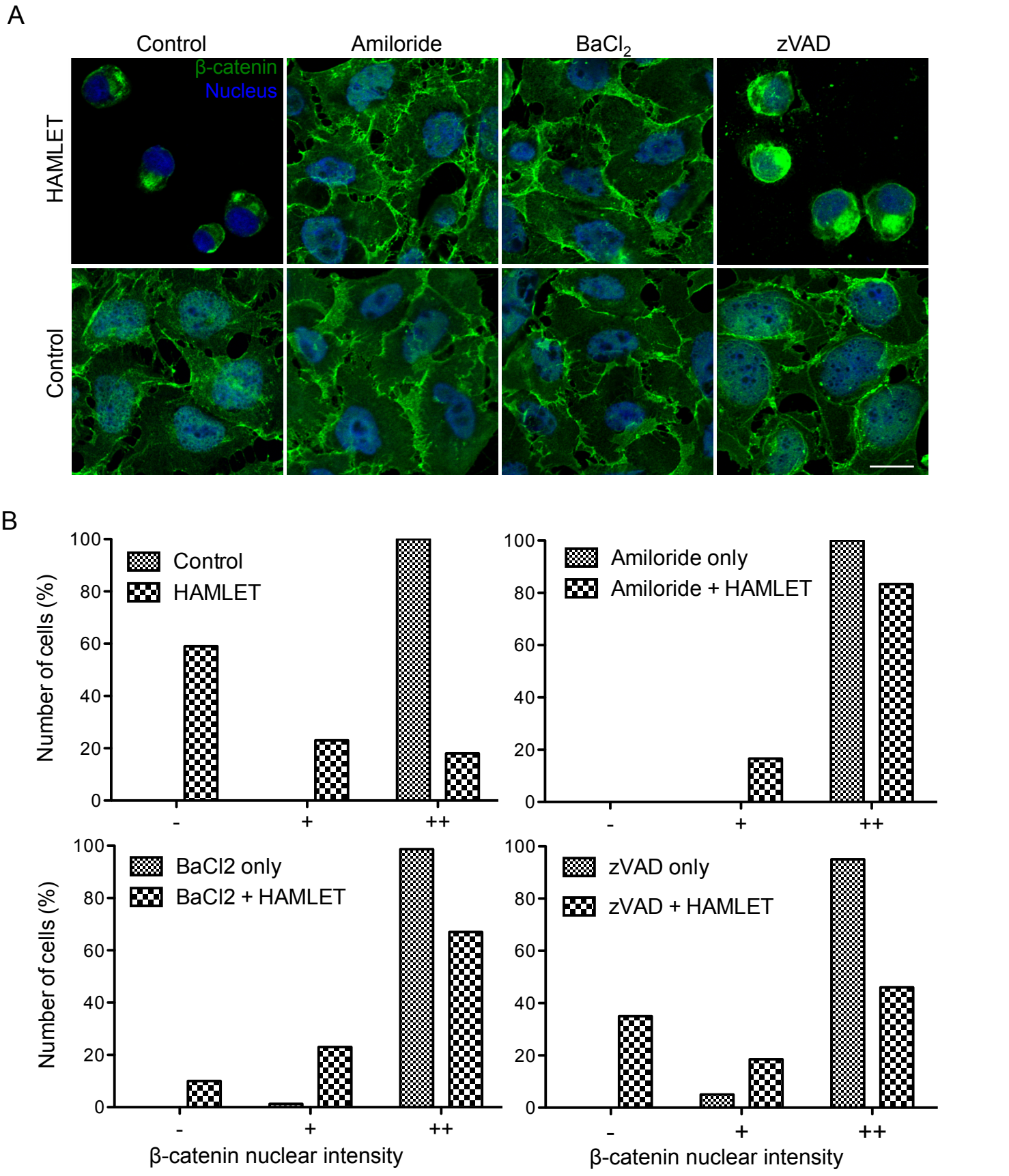
Supplementary Figure 8



Supplementary Figure 8. Ion channel inhibitors prevent HAMLET-induced loss of nuclear β-catenin. (**A**) HAMLET-induced (35 μM, 3 h) loss of nuclear β-catenin staining (green) was significantly prevented by pretreatment of DLD1 cells with ion channel inhibitors (Amiloride and $BaCl_2$, 1 mM). Caspase inhibitor zVAD (50 μM) only partially prevented HAMLET-induced loss of nuclear β-catenin. Nuclei are stained blue with DRAQ-5 (scale bar, 10 μm). (**B**) Histograms showing quantification of nuclear β-catenin staining. Staining was recorded on a 3-point scale: -, cells with no nuclear β-catenin staining; +, cells with low nuclear β-catenin staining; ++, cells with high nuclear β-catenin staining (280-320 cells were counted for each panel. *n* = 3, Scoring by two independent observers).

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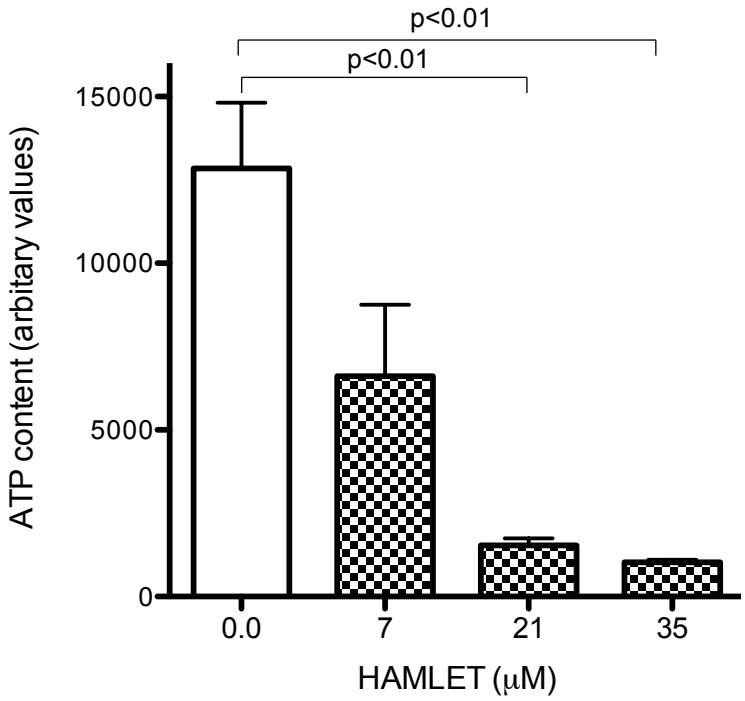
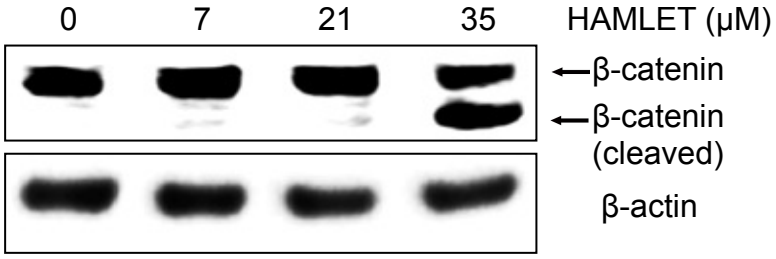
Supplementary Figure 9

A



B



Supplementary Figure 9. Effects of HAMLET on human colon cancer cells SW480. (**A**) Dose dependent loss of viability after HAMLET treatment (3 hours), quantified by ATP measurements (*n* = 2). (**B**) Western blot showing HAMLET induced degradation of β-catenin in SW480 cells. Cells were cultured overnight in 6 well plates and treated with HAMLET for 3 hours. β-actin was used as a loading control (*n* = 2).

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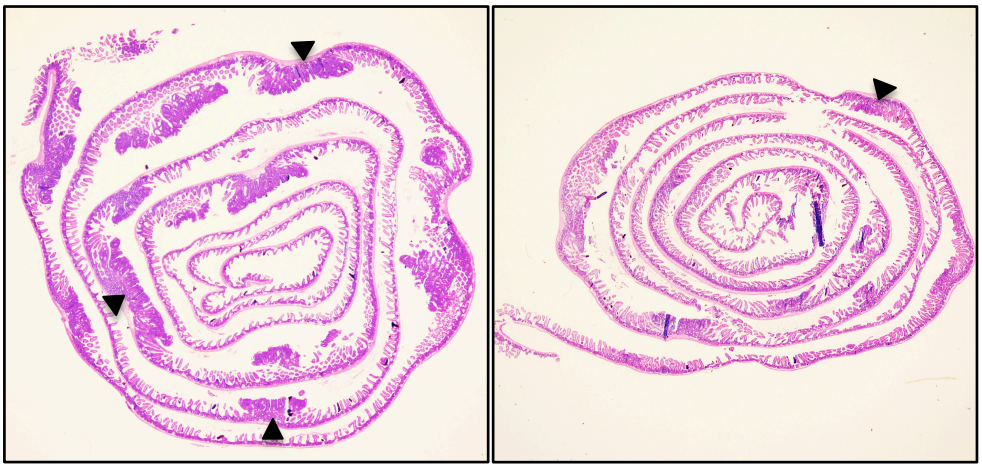
Supplementary Figure 10



Supplementary Figure 10. HAMLET prophylaxis reduces tumor burden in APC$^{Min/+}$ mice. Representative H&E staining of intestinal ‘‘Swiss roll’’ section showing a decrease in polyp number and size after HAMLET prophylaxis (arrowhead, polyps).

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Supplementary Figure 11

Gene sets suppressed by HAMLET

Gene set	FDR q-val
DNA Replication	0.017
WNT/Beta Catenin Pathway	0.063
Cell Communication	0.064
Ribosome	0.068
ECM Receptor Interaction	0.266

Gene sets enriched by HAMLET

Gene set	FDR q-val
Glutathione Metabolism	0.000
Oxidative Phosphorylation	0.001
TCA Cycle	0.042
Glycolysis	0.096
Butanoate metabolism	0.150

Supplementary Figure 11. Top five most significant gene sets enriched or depleted in HAMLET-treated compared to sham-treated APC$^{Min/+}$ mice tumors. Pathway analysis of differentially expressed genes using GSEA indicated highly significant enrichment of Glutathione Metabolism, Oxidative Phosphorylation, TCA Cycle and Glycolysis pathways after HAMLET-treatment. DNA Replication, WNT/β-catenin Pathway, Cell Communication and Ribosome pathways were more highly expressed in mock-treated animals.

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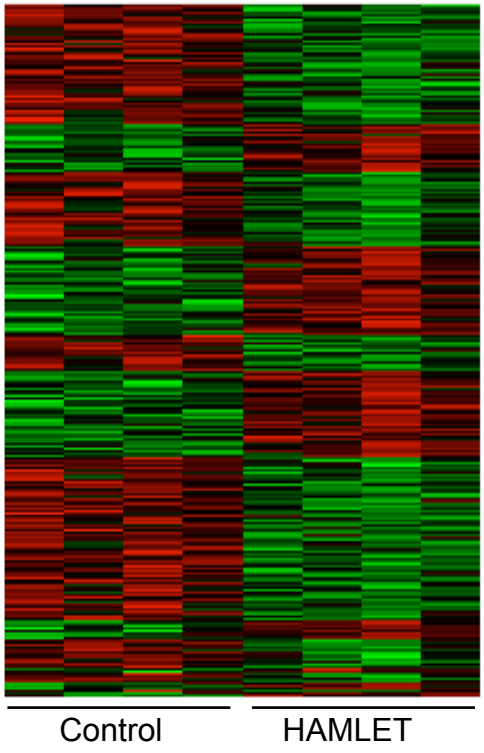
Supplementary Figure 12

A



B

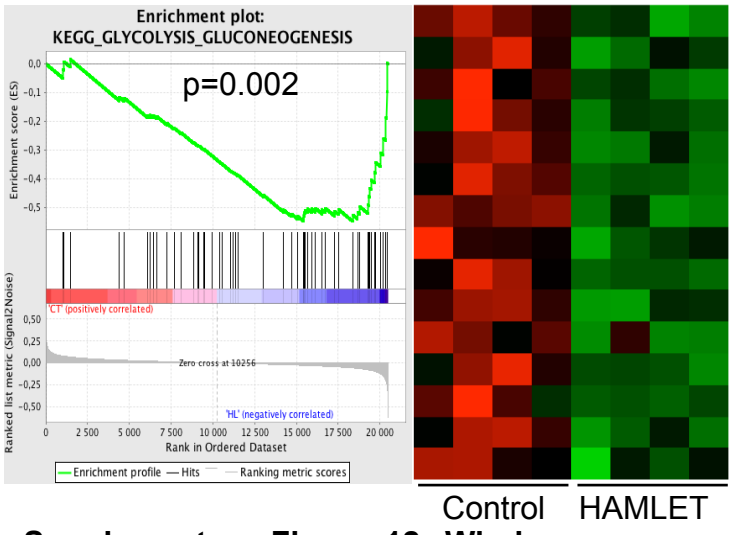
Gene Set Enrichment Analysis

Gene set	FDR q-val
KEGG Retinol Metabolism	0.017
WNT Signalling	0.032
KEGG Hedgehog Signalling Pathway	0.05
KEGG ECM Receptor Interaction	0.05
KEGG Glycolysis / Glyconegenesis	0.05
KEGG Glutathione Metabolism	0.043

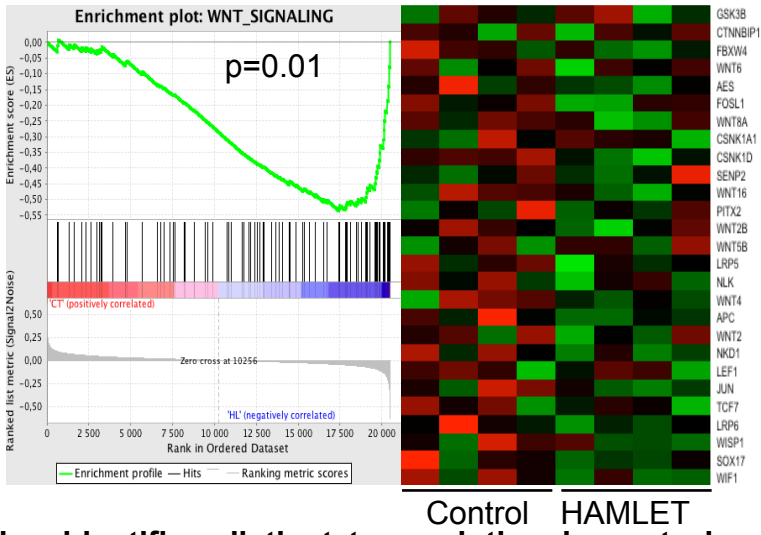
Ingenuity Pathway Analysis

Canonical Pathway	-log(p-value)
Glycolysis/Gluconeogenesis	9.36
Fructose and Mannose Metabolism	6.05
Metabolism of Xenobiotics by Cytochrome P450	5.78
LPS/IL-1 Mediated Inhibition of RXR Function	5.46
Arachidonic Acid Metabolism	5.17
Fatty Acid Metabolism	4.71

C



D



Supplementary Figure 12. Whole genome profiling identifies distinct transcriptional events in HAMLET-treated tumors. Five weeks after HAMLET-treatment, existing tumors in treated and sham groups were excised and total RNA was extracted and hybridized to Affymetrix MG430 whole genome arrays. (**A**) Heatmap showing the top 500 differentially expressed genes between HAMLET and mock treated, indicating that there are distinct differences between the two groups. (**B**) Pathway analysis of differentially expressed genes using GSEA or Ingenuity Pathway Analysis. Highlighted WNT signaling and Glycolytic pathway as enriched in HAMLET-treated tumors. The most highly expressed genes in HAMLET treated tumors were involved in glucose metabolism (Ingenuity *P*-value = 10^{-9} and GSEA FDR q-value = 0.05) and upregulated genes included a large fraction of key enzymes responsible for glycolytic fluxes (phosphoglucomutase 1, phosphofructokinase, enolase, pyruvate kinase M2 and alcohol dehydrogenase). GSEA analysis confirmed the high expression of glycolytic enzymes. In addition, genes associated with the inhibition of WNT signaling were enriched in the HAMLET-treated tumors (q-values 0.032). WNT inhibitory factor 1, a secreted protein containing a WNT inhibitory factor (WIF) domain and 5 epidermal growth factor (EGF)-like domains [14] was upregulated two-fold in HAMLET treated tumors as was SOX17 (SRY (sex determining region Y)-box 17), which modulates WNT signaling through binding to WNT3A. Other upregulated genes included JUN, consistent with innate immune activation by HAMLET in healthy cells (Storm et al., Manuscript). (**C-D**) GSEA results showed that the Glycolytic pathway and WNT pathway are enriched in HAMLET treated tumors.

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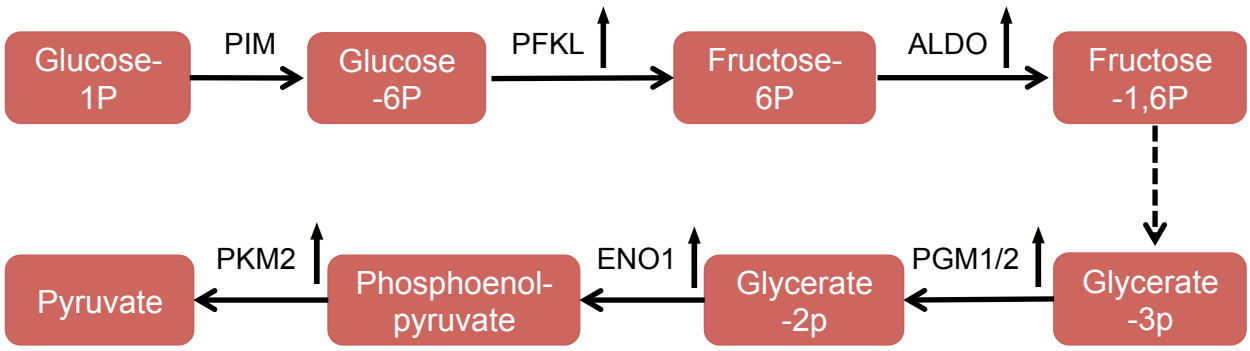
Supplementary Figure 13



Supplementary Figure 13. Schematic representation of metabolites (red boxes) and enzymes that were upregulated in HAMLET-treated tumors.

Supplementary Methods

Chemicals

Amiloride, Barium chloride, Leupeptine, Formaldehyde, Triton X-100, Tween-20, Sodium dodecyl sulphate (SDS), Sodium deoxycholate and Fluoromount were from Sigma (St. Louis, MO). EDTA (ethylenediaminetetraacetic acid) and Tris (hydroxymethyl) aminomethane were from VWR (Volumetric solutions, BDH Prolabo). zVAD was purchased from Biomol and DRAQ-5 from eBioscience.

Cell Culture and cell death assays

DLD1 cells were procured from American Type Culture Collection (ATCC) and were maintained in RPMI-1640 medium supplemented with 1 mM sodium pyruvate (Fisher Scientific), non-essential amino acids (1:100) (Fisher Scientific), 50 µg/ml gentamicin (Gibco, Paisley, UK) and 10% fetal calf serum (FCS, PAA Laboratories GmbH). Cells were cultured at 37°C temperature, 90% humidity and 5% CO_2. Cells were grown in 96 well plates overnight (for cell death assays), in 6 well plates (for western blots), and in 75 mm flasks (for immunoprecipitation). ATP levels were measured by ATPlite Kit (PerkinElmer) and cell viability was quantified by PrestoBlue cell viability reagent (Invitrogen, Molecular Probes) following manufacturer's instructions.

Immunohistochemistry

Swiss rolls of longitudinally opened intestinal segments were fixed overnight in 10% neutral buffered formalin. Samples were embedded in paraffin and 5µm thick sections were further processed for immunohistochemistry using antibodies for β-catenin (BD Biosciences), cyclin D1 (Thermo Scientific), Ki-67 (Thermo Scientific), VEGF (Santa Cruz) and COX-2 (Cayman chemical). For immunocytochemistry, DLD1 cells were grown overnight on 8-chamber glass slide (Lab-Tek, Chamber Slide, Thermo Fisher Scientific) at a concentration of $5x10^4$ cells per well. After respective treatments, cells were fixed with paraformaldehyde, permeabilized (0,1 % Triton X-100 in 5 % FCS-PBS, 10 min) and blocked (10 % FCS, 1 h). Subsequently, cells were incubated with anti β-catenin antibody (1:50 dilution, BD Bioscience) or with anti E-cadherin (1:200, R&D) antibody overnight at 4°C. Secondary antibodies were labeled with Alexa Fluor 448 or Alexa Fluor 568 (1:200, 1 h, Molecular Probes). DRAQ-5 (Bioscience, San Diego, CA) was used as nuclear stain (1:500, 5 min). Cells were mounted with mounting media (Fluoromount, Sigma) and Fluorescence was detected in a LSM510 META confocal microscope (Carl Zeiss, Germany).

Western blots

Tumors and healthy intestinal tissue was collected and kept at -80ºC. From *in vivo* experiments, tissue lysates were prepared in 2% SDS buffer. From *in vitro* experiments, cell lysates were prepared in in RIPA lysis buffer. After SDS-PAGE, proteins were electroblotted to a PVDF membrane. Membranes were than blocked and incubated with primary antibodies including β-catenin (1:4000-1:20,000, BD Biosciences), β-actin (1:2000-1:10,000, Sigma), GSK 3β (1:1000, Cell Signaling), p-GSK 3α/β (1:1000, R&D), Cleaved caspase 3 (1:500, Cell Signaling), E-cadherin (1:5000, R&D), Cyclin D1 (1:1000, Thermo Scientific) followed by secondary antibody incubation including Goat anti- rabbit HRP (1:2000, Santa Cruz) and Mouse IgG (1:5000, DAKO) and visualized using ECL (Amersham Biosciences, Piscataway, NJ).

Measurement of COX-2 activity and VEGF

COX-2 activity was measured according to the manufacturer's instructions (Cayman Chemicals). Briefly, samples were homogenized in ice-cold lysis buffer (0.1M Tris-HCl, pH 7.5, supplemented with protease inhibitor mixture (Sigma-Aldrich)) for 20 seconds. The homogenates were centrifuged (10,000 x g, 15min, 4°C), the supernatants collected and stored at -80ºC for subsequent analysis. The results were corrected for the DNA content of the tissue sample and expressed as fold increase over control. For VEGF ELISA, intestinal tumor tissue was rinsed with PBS, homogenized and stored at -20^{0}C. Two freeze-thaw cycles were performed to break cell membranes and homogenates were centrifuged (5,000 x g, 5 min, 4°C) and stored at -80ºC for subsequent analysis. ELISA was performed according to manufacturer's instructions (Quantikine Mouse VEGF, R&D Systems), cut off >3.0 pg/ml.

Immunoprecipitation

After treatment with HAMLET, cells were lysed using Qproteome® mammalian protein prep kit (Qiagen) for 30 min and then centrifuged at 12,000g at 4 °C for 10 min. The supernatants were collected and incubated with protein A/G beads (Invitrogen) for 1 hour at 4 °C. After preclearing, the lysate was incubated with Mouse E-cadherin antibody (1:100, R&D Systems) overnight at 4 °C followed by incubation with protein A/G beads for overnight at 4 °C. The complexes thus formed were washed two times with cell lysate containing protease inhibitor. After washing the bead-protein complexes were boiled in equal volume of 2x SDS sample buffer (Invitrogen) and used for Western blot using anti-E-cadherin antibody (1:2000, R&D Systems) or anti-β-catenin (1:2000 to 1:5000, BD Biosciences).

Intracellular ion concentrations and ion fluxes

The relative free intracellular Ca^{2+} ion concentration was measured using calcium indicator Fluo4 NW (Invitrogen). Briefly, 20,000 adherent cells (DLD1) were incubated with Fluo4 NW (5μM) in assay buffer at 37°C for 45 min. Cells were subsequently pretreated with ion channel inhibitors (1mM) followed by treatment with HAMLET (35μM). The Ca^{2+} ionophore A23187 (1μM, invitrogen) was used as positive control (data not shown). For estimation of K^+ fluxes, the FluxOR™ potassium ion channel assay (Invitrogen) was used. An opening of K+-channel corresponds to an influx of Ti+, which act as an indicator of K+-channel opening. Briefly, 20,000 adherent cells were incubated in loading buffer followed by its incubation with assay buffer and stimulus buffer for 60 min. Cells were pretreated with ion channel inhibitors (1mM) and than treated with HAMLET (35μM). Nigericin (1μM) was used as a positive control (data not shown). Both Fluo4 NW and and FluxOR™ fluorescence were measured at 535 nm after excitation at 485 nm using fluorescence plate reader (TECAN infinite F200, Tecan Group, Switzerland).

Transcriptomic analysis

For the *in vivo* transciptomics analysis, tumors tissues were collected at the end of treatment in the prophylactic study and five weeks after the end of treatment in the therapeutic study. Tissues were stored in RNAlater (Ambion) at -80ºC for subsequent analysis. Approximaetly 5 mg of tissue was homogenized using a Tissuelyser (Qiagen) and total RNA was extracted using RNeasy (Qiagen). For transcriptomic analysis, 300 ng of total RNA was converted into cRNA using Affymetrix 3' IVT Express Kit as per manufacturers instructions. 10 µg of labeled cRNA were hybridized to Affymetrix MG-430 PM Array Strips. After hybridization and washing, the fluorescence intensity was measured in the Affymetrix Gene Atlas system. Array data were normalized using the robust multichip average (RMA) and filtered for expression values lower than 2^5. Differentially expressed genes were identified, by fitting the normalized data to a linear model and genes with a FDR-adjusted p-value < 0.05 and log 2 foldchange > 1 was considered differentially expressed. To identify functionally relevant alterations, the gene list was submitted to Ingenuity Pathway Analysis. Gene set-enrichment analysis (GSEA) was performed by the use of the Canonical Pathways Geneset collection v. 2.5 (http://www.broadinstitute.org/gsea/msigdb/index.jsp). The microarray dataset reported here has been deposited in NCBI's Gene Expression Omnibus and will be accessible at time of publication.

***In vitro* transcriptomics**

For the *in vitro* transcriptomics, DLD-1 cells were seeded at a density of 150000 cells/well in six-well plates. After overnight incubation, the cells were treated with HAMLET (35 µM), oleic acid (Sigma, 175 µM) or a-lactalbumin (35 µM) for 3 hours. Total RNA was extracted using RNeasy (Qiagen) and purity and quantity was determined on a Nanodrop. 300 ng of totalRNA was used for generation of labeled cRNA and hybridized to Affymetrix HGU219 microarray strips on the Affymetrix Geneatlas System. Raw data was normalized using RMA and further analyzed using R/Bioconductor. Genes with a log2-foldchange > 1 and FDR-adjusted p-value < 0.05 were considered differentially expressed.