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Supplemental Information

Caloric Restriction Mimetics Enhance Anticancer Immunosurveillance

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Supplemental Data

Table S1, related to Figure 1, Figure 3, Figure 4, Figure 5 and Figure 6. (Provided as Excel File) Statistical significance for cross-sectional analyses calculated by ANOVA Test for Multiple comparisons.

Table S2, related to Figure 1, Figure 3, Figure 4, Figure 5 and Figure 6 (Provided as Excel File) Statistical significance of longitudinal trend for tumor growth experiments calculated by ANOVA Type 2 (Wald's Test) for pairwise comparison

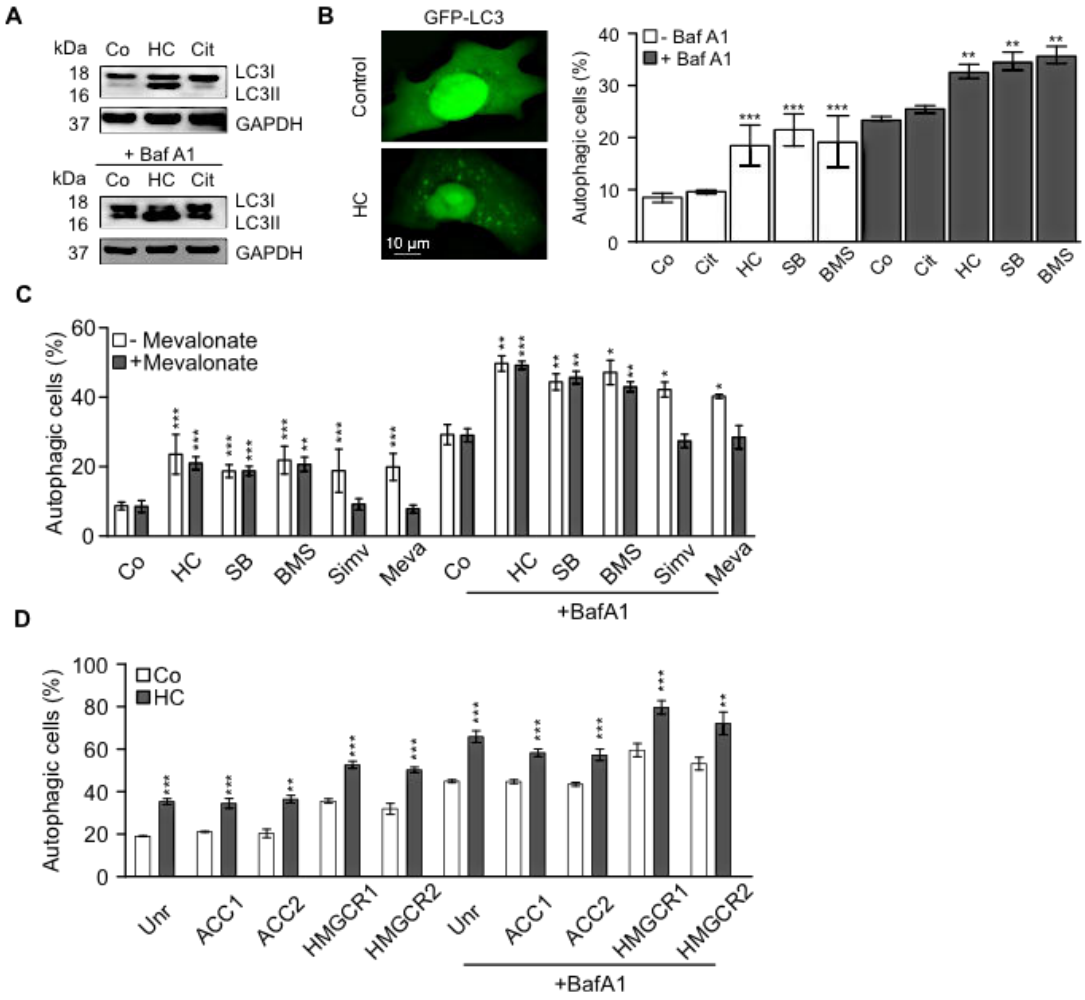

Figure S1, related to Figure 1. Hydroxycitrate-induced autophagy is independent of fatty acids and sterols biosynthetic pathways. (A) Representative immunoblot depicting the autophagy-related LC3I to LC3II conversion induced by hydroxycitrate (HC) but not citrate (Cit) in human U2OS osteosarcoma cells in the presence and absence of bafilomycin A1 (BafA1). **(B)** Stimulation of autophagic puncta by HC, SB204990 (SB) and BMS303141 (BMS), alone or together with BafA1, in U2OS cells stably expressing green fluorescent protein (GFP)-LC3. Representative microphotographs (**left panel**) and quantification (**right panel**) as means ± SEM from three different experiments. **(C)** Human U2OS osteosarcoma cells stably expressing a GFP-LC3 fusion protein were treated for 12 h with ATP citrate lyase (ACLY) inhibitors or the hydroxymethylglutaryl CoA reductase (HMGCR) inhibitors simvastatin (Simv) and mevastatin (Meva), in the presence or absence of Mevalonate. Results (means ± SEM) from three different experiments **(D)** Human U2OS osteosarcoma GFP-LC3 cells were transfected with unrelated control siRNA (Co) or with two different siRNAs targeting Acetyl CoA Carboxylase (ACC) or HMGCR. Results (mean ± SEM) from three different experiments. Statistical analysis was performed by Student's *t* test in comparison to the control condition. Level of significance, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

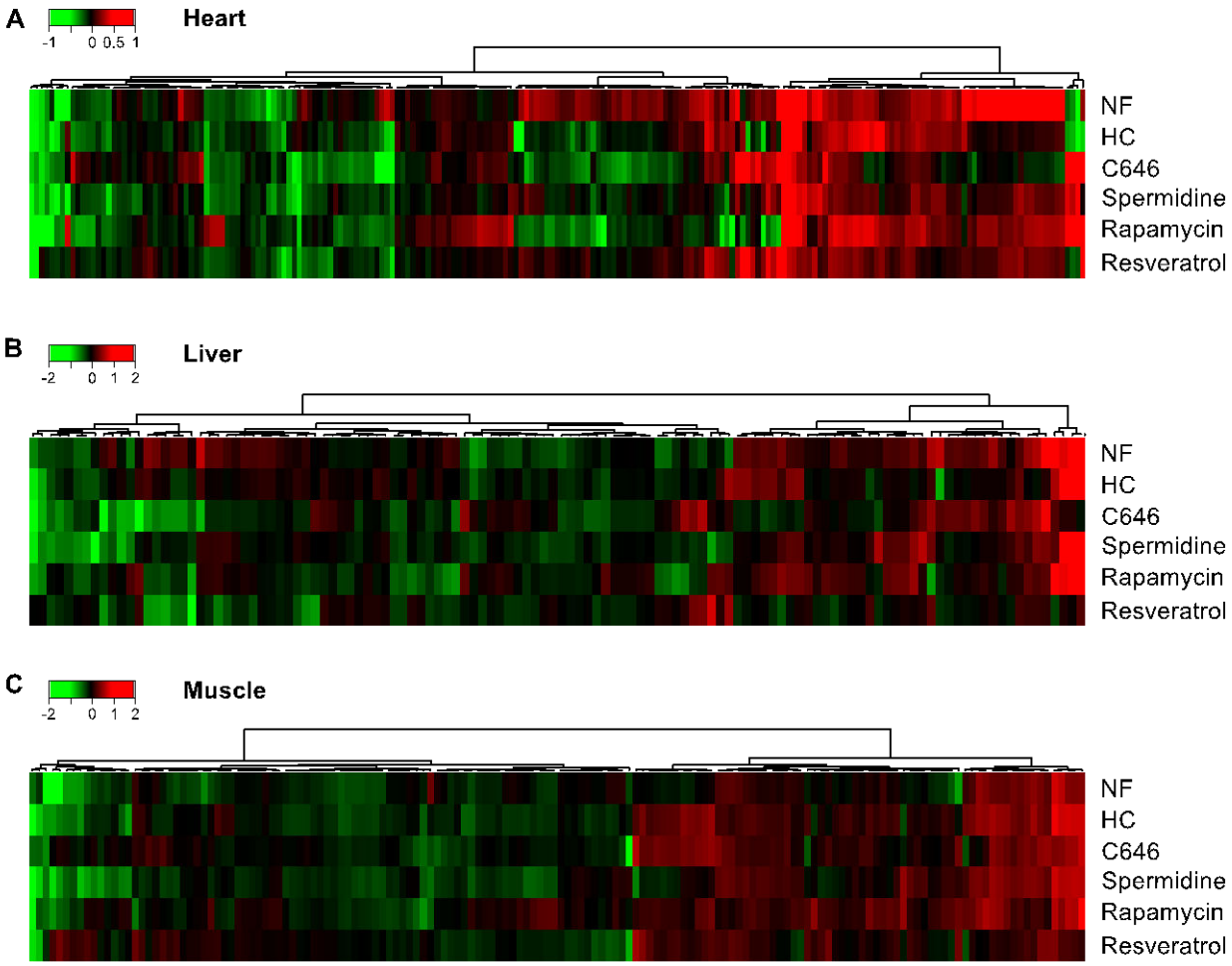

Figure S2, related to Figure 2. Fasting and Caloric Restriction Mimetics administration induce convergent metabolic perturbations. (A-C) Heatmap depicting log2 fold changes to the control of metabolite signals found significantly altered in heart **(A)**, liver **(B)** or muscle **(C)** after 48 h starvation or two injections of the indicated CRMs. Values are expressed as log-basis 2 fold-change compared to untreated controls. A comprehensive list of metabolites is indicated in **Table S3**.

Table S3, related to Figure 2 (Provided as Excel File). List of metabolites detected by untargeted analysis of intracellular metabolites by UHPLC after univariate analysis. Table depicting Fold Change (FC) and p- values compared to Control Conditions in different mouse organs.

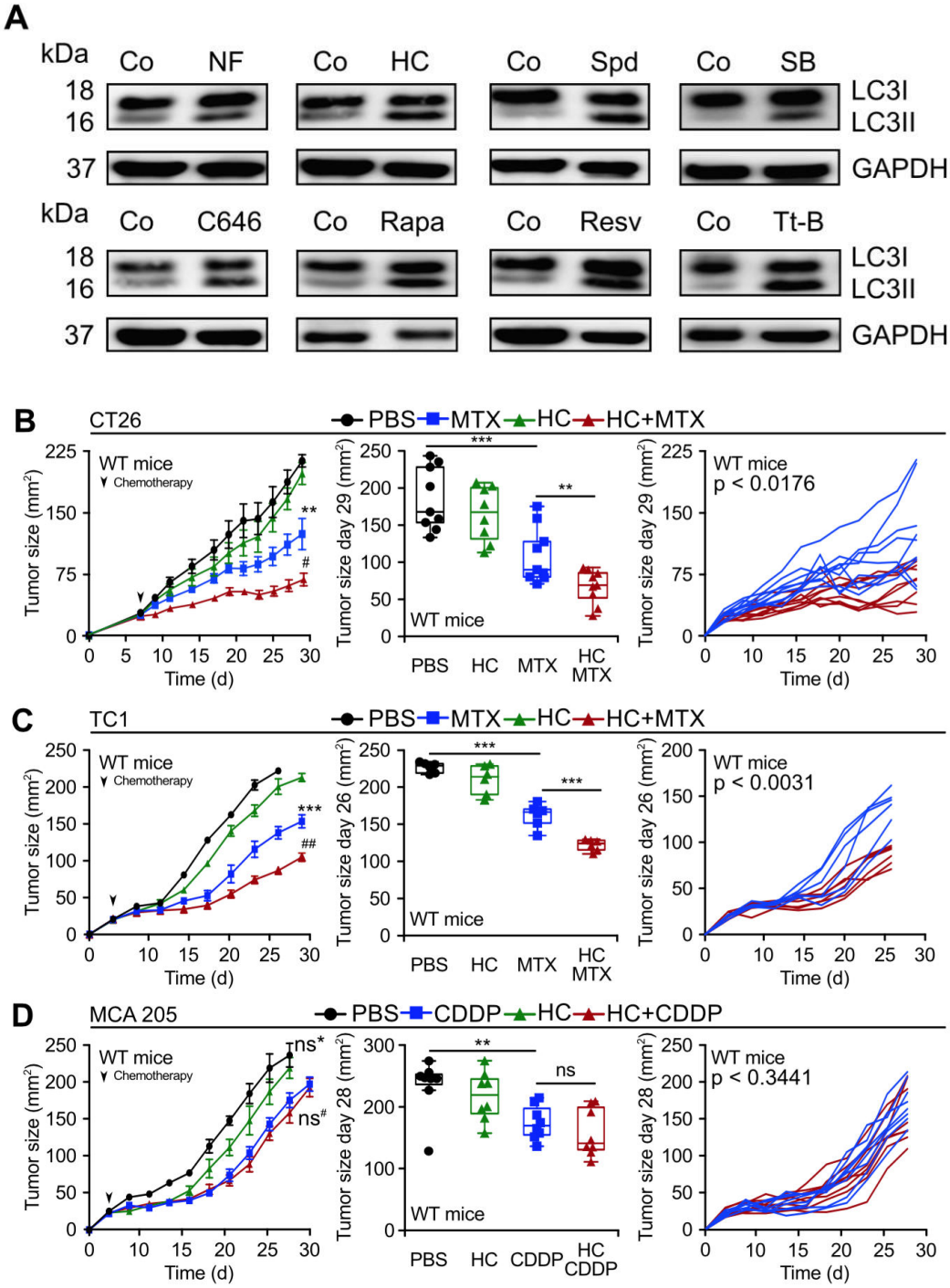

Figure S3, related to Figure 3 and Figure 4. Hydroxycitrate slows tumor growth in various tumor models. (A). Effects of autophagy inducers on MCA205 fibrosarcomas *in vivo*. Different caloric restriction mimetics and canonical autophagy inducers trigger autophagic response in murine MCA205 fibrosarcoma derived tumors. Representative immunoblots depicting LC3I to LC3II conversion in MCA205 tumors after 48 h fasting (NF) or hydroxycitrate (HC), spermidine (Spd), SB204990 (SB), C646, rapamycin (Rapa), resveratrol (Resv) or the pro-autophagic Tat-Beclin 17 (Tt-b) peptide. (**B**) Wild type (WT) immunocompetent Balb/c mice were inoculated *s.c.* with murine colorectal cancer CT26 cells. When tumors became palpable, mice received systemic HC administration in drinking water, alone or in combination with MTX. **(C)** WT immunocompetent C57Bl/6 mice were inoculated *s.c.* with murine lung cancer TC1 cells. When tumors became palpable, mice received systemic HC administration in drinking water, alone or in combination with MTX. Results in **B,C,D** (averaged tumor growth curves ± SEM) are plotted and statistical calculations are performed as previously described (**D**) Effect of hydroxycitrate combined with a non-immunogenic anticancer chemotherapy. Wild type (WT) immunocompetent C57BL/6 mice were inoculated *s.c.* with murine fibrosarcoma MCA205 cells. When tumor reached around 25 mm^2 surface, mice were administered with HC in drinking water. At day 2 post HC administration, mice received chemotherapy with cisplatin (CDDP) or an equal volume of phosphate buffer saline (PBS). Results are plotted and statistical calculations are performed as previously described.; ** $p < 0.01$; *** $p < 0.001$; (PBS *vs* chemotherapy); $^{\#}p < 0.05$; $^{\#\#}$p value < 0.01 (chemotherapy *vs* CRMs + chemotherapy). A comprehensive account of all comparisons is indicated in Table **S1** and **S2**.

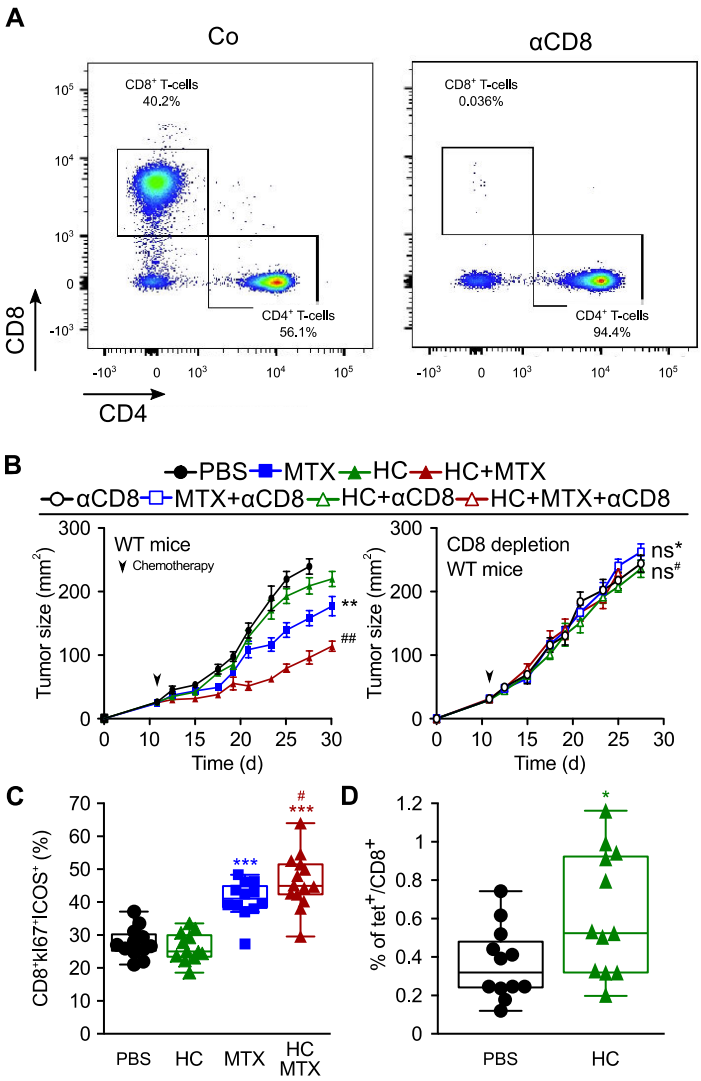

Figure S4, related to Figure 3 and Figure 4. Hydroxycitrate triggers an antitumor CD8-dependent immune response. (A,B) (**A**) FACS profile showing the effective depletion of Antibody-mediated depletion of $CD8^+$ T lymphocytes abolishes the anticancer effects of HC. (**B**) Wild-Type (WT) immunocompetent C57BL/6 mice were *s.c.* inoculated with murine fibrosarcoma MCA205 cells. When tumors became palpable, mice were administered with hydroxycitrate (HC) in drinking water and were *i.p.* injected with control isotype or αCD8 antibody. At day 2 post HC administration, mice were treated by Mitoxantrone (MTX). Data are depicted as means ± SEM. ** p value < 0.01 (Wald's Test, PBS *vs* MTX); $^{\#\#}$ p value < 0.01 (Wald's Test, MTX *vs* HC+MTX); ns non-significant. Panel of MCA205 treated with control isotype is repeated in **Suppl. Fig 6** since PBS, MTX and HC treated groups are shared with αCD73 depletion experiment. (**C**) Frequency of $CD8^+$ $Ki67^+$ $ICOS^+$ cells in tumor infiltrate. *** p < 0.001 (ANOVA Test for multiple comparison, PBS *vs* MTX and PBS *vs* HC + MTX); $^{\#}$ *p* < 0.05 (ANOVA Test for multiple comparison, MTX *vs* HC + MTX). (**D**) **Hydroxycitrate enhances the frequency of TC1-specific HPV16-E7 tetramer$^+$ CD8$^+$ T cells in the draining lymph node of TC1 tumors.** C57BL/6 mice were inoculated *s.c.* with murine lung cancer TC1 cells. When tumors became palpable, mice were administered with HC. At day 4 post-HC administration, mice were *i.p.* injected with 20 µg of Shiga toxin B subunit and 0.01 µg/µL of galactosyl ceramide (GalCer). At day 28, draining lymph node (dLN) were analyzed for the presence of TC1-specific HPV16-E7 tetramer$^+$ $CD8^+$ T cells. *p < 0.05 (unpaired Student's *t* test).

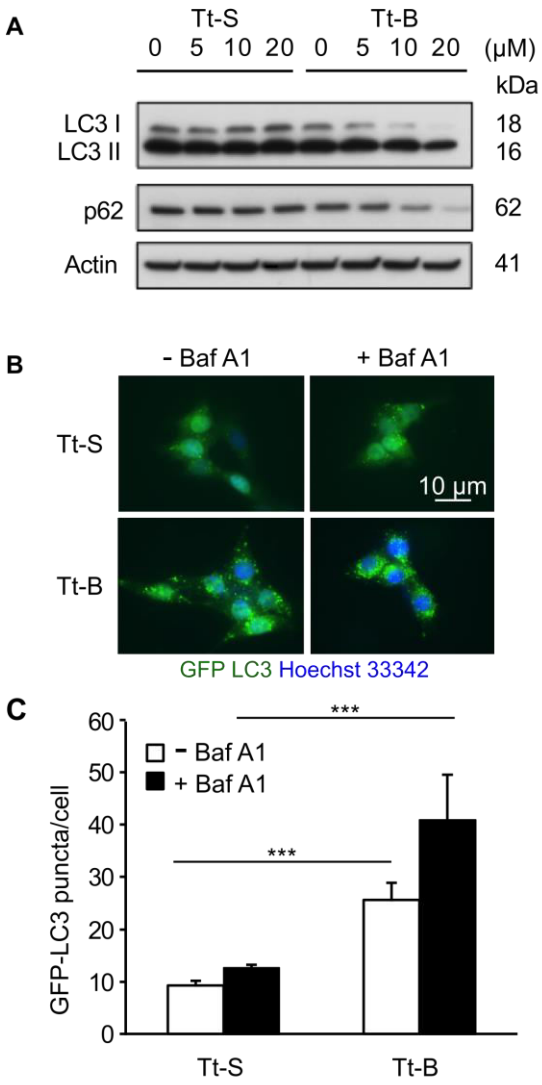

Figure S5, related to Figure 4. Tat-Beclin 1 peptide, but not its mutated counterpart, stimulates autophagy *in vitro*. Tat-Beclin1 (Tt-B) derived peptide 17 triggers autophagy *in vitro* in murine fibrosarcoma MCA205 cells. **(A)** Representative immunoblotting showing autophagy induction mediated by 3 h incubation of MCA205 fibrosarcoma cells in presence of Tat-Beclin peptide 17 (Tt-B) and its inactive form Tat-Beclin peptide 17 (Tt-S). Growing concentrations of Tt-B, but not of Tt-S, triggers autophagy as depicted by LC3I to LC3II lipidation and by degradation of the autophagic target p62. **(B,C).** Representative images **(B)** and corresponding quantification **(C)** of MCA205 cells stably expressing GFP-LC3 after 3 hours treatment with 10 μM of Tt-B and Tt-S. Autophagic flux was monitored in presence or absence of BafilomycinA1. Data are depicted as means ± SD. *** $p < 0.001$ (unpaired Student's *t test*) compared to Tt-S.

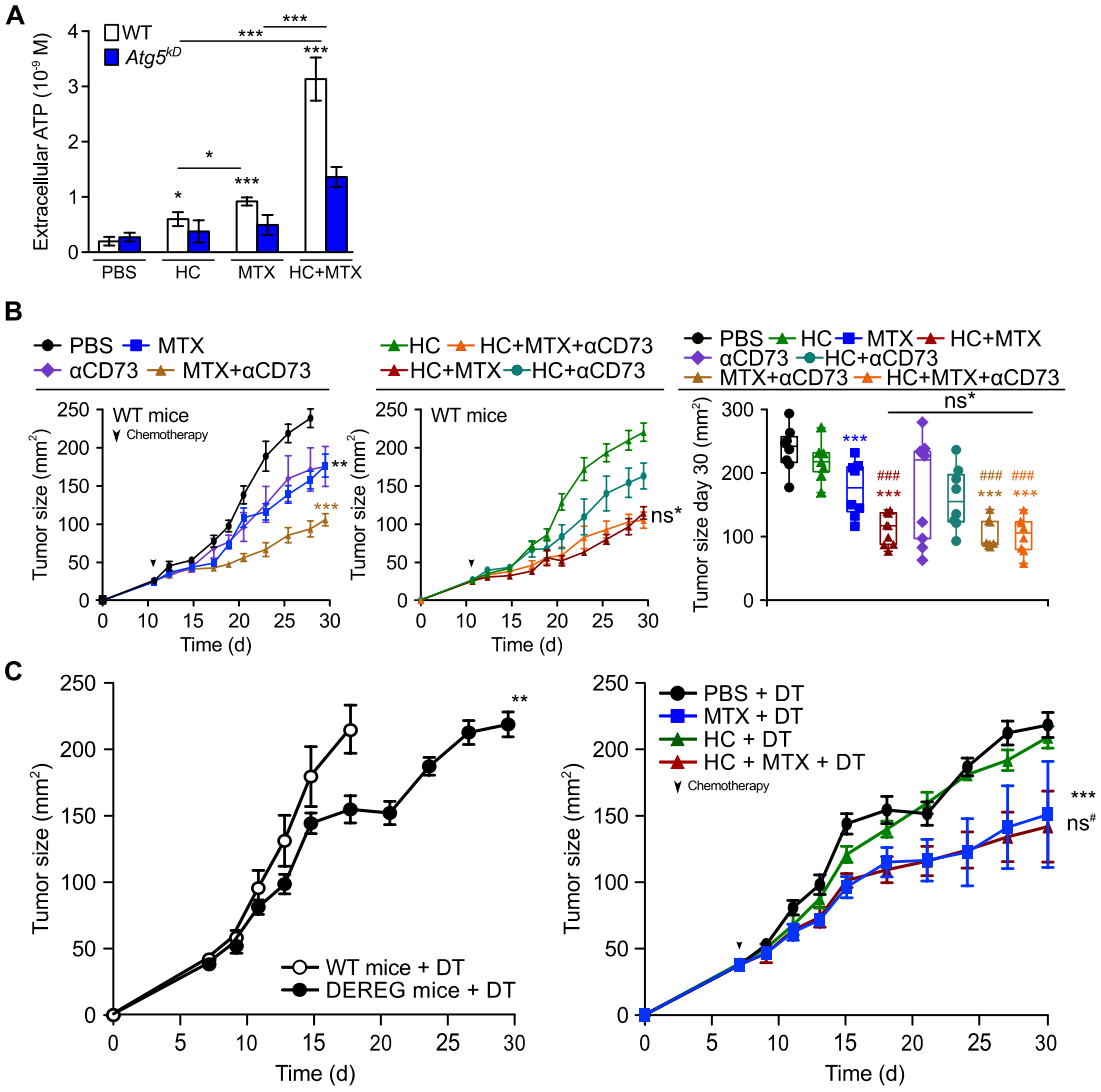

Figure S6, related to Figure 6. Hydroxycitrate anti-cancer effect relies on ATP mediated Tregs recruitment to tumor bed. **(A)** Autophagy-dependent release of ATP *in vitro*. Autophagy-competent or *Atg5*^{KD} MCA205 cells were treated with hydroxicitrate (HC) and/or mitoxantrone (MTX), and ATP was measured in the supernatants. Results (mean ±SEM) from two different experiments. If not indicated by lines, comparisons are referred to Co. * $p < 0.05$, *** $p < 0.001$ (ANOVA Test for multiple comparison). (**B**) HC-mediated anti cancer effects are epistatic to CD73 inhibition. Wild-Type (WT) Immunecompetent C57BL/6 mice were *s.c.* inoculated with murine fibrosarcoma MCA205 cells. When tumors became palpable, mice were administered with HC in drinking water and were *i.p.* injected with control isotype or αCD73 antibody. At day 2 post HC administration, mice were treated by MTX. Data (mean ±SEM, one experiment with eight mice/group) are shown and analyzed as previously described. Statistical analysis was performed by linear mixed effect modeling (over the whole time course) and linear modeling (at a single time point). Level of significance: ** $p < 0.01$ (Wald's Test, PBS *vs* MTX); *** $p < 0.001$ (Wald's Test, PBS *vs* MTX+ aCD73). For single time point analysis at day 30, *** $p < 0.001$ (compared to PBS); $^{###}$ $p < 0.001$ (compared to MTX). A comprehensive account of all comparisons is indicated in Table **S1** and **S2**. (**C**) **Effects of the depletion of regulatory T cells (Tregs) by diphteria (DT) toxin on TC1 tumors treated with HC.** C57BL/6-Tg (Foxp3-DTR/EGFP) DEREG (DEpletion of REGulatory T cells) transgenic mice and their wild type littermates were inoculated *s.c.* with murine lung cancer TC1 cells (**A,B**). When tumors became palpable, mice were treated daily with HC in drinking water and received *i.p.* injection of 1µg/kg diphteria toxin (DT) for 15 days . At day 2 post DT and HC administration, DEREG mice received chemotherapy with mitoxantrone (MTX) or Phosphate Saline Buffer (PBS). Results are shown as means ± SEM (at least 8 mice per group). ** p value < 0.01 (Wald's Test); *** p value < 0.001 (Wald's Test). Ns, non significant.

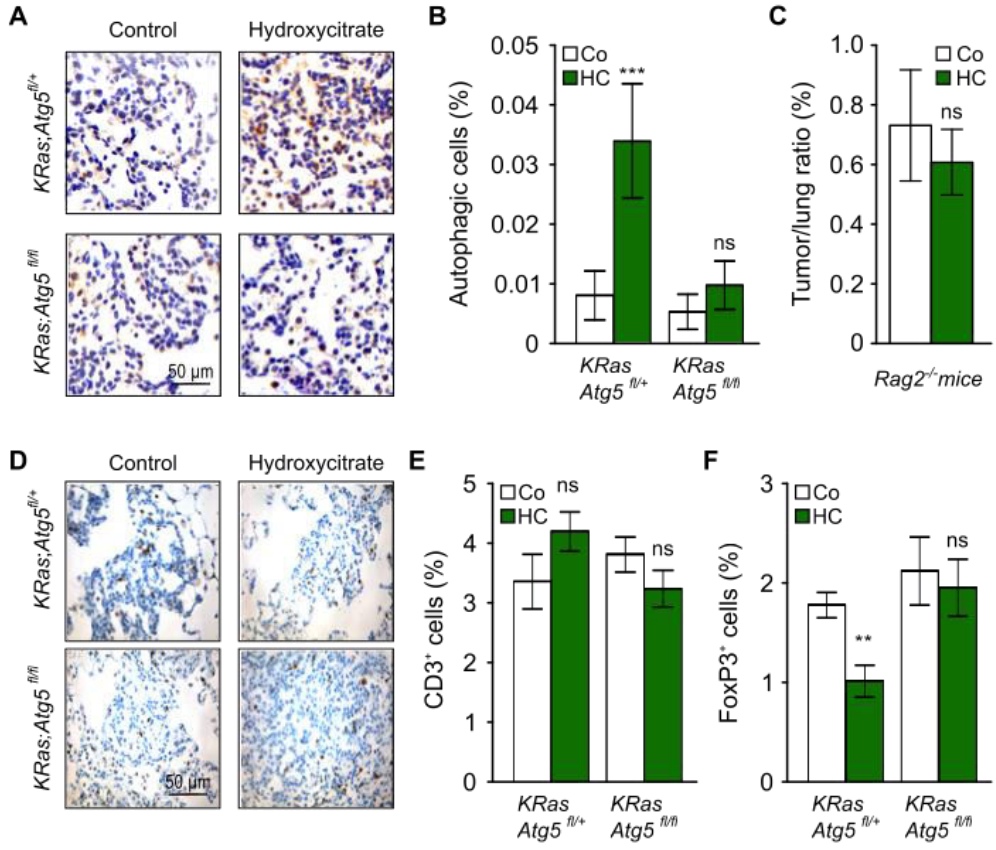

Figure S7, related to Figure 7. Hydroxycitrate (HC) induces autophagy in *KRas;Atg5*$^{fl/+}$ mouse lung tissues. **(A)** Immunohistochemistry staining of LC3B in *KRas;Atg5*$^{fl/+}$ compared to *KRas;Atg5*$^{fl/fl}$ mice lung tissues. Quantification is depicted in **(B)**. Results are shown as means ± SD. ns, non significant; *** $p < 0.001$ (unpaired Student's *t test*), as compared to control conditions. **(C)** Effect of HC on *KRas*-induced lung cancer relies on adaptive immunity. HC failed to significantly reduce neoplastic lesions in *Rag2*$^{-/-}$ mice. Results are shown as means ± SD; ns, non significant (unpaired Student's *t* tes*t*), as compared to control conditions. (**D,E**) Representative histological sections of $CD3^+$ stained T cells (**D**) after 5 weeks of HC administration and corresponding quantification (**E,** mean ± SEM, three independent experiments). (**F**) Quantification (mean ± SEM, three independent experiments) of $FoxP3^+$ stained cells shown in Fig. **7C.** Level of significance: ** $p < 0.001$ (unpaired Student's *t* test); ns, non significant;

Supplemental Experimental Procedures

Chemicals and culture conditions. Unless otherwise indicated, media and supplements for cell culture were purchased from Gibco-Invitrogen Life Technologies Inc. (Carlsbad, CA, USA) while plasticware was purchased from Corning B.V. Life Sciences (Amsterdam, The Netherlands). Human osteosarcoma U2OS and their green fluorescent protein (GFP)-LC3-expressing derivatives (gift from Professor J. Yuan, Harvard University), were cultured in DMEM medium supplemented with 10% (v/v) fetal bovine serum, 100 mg/L sodium pyruvate, 10 mM HEPES buffer, 100 IU mL^{-1} penicillin G sodium salt, and 100 mg/mL streptomycin sulfate. All cells were maintained in standard culture conditions (at 37 ºC, under 5% CO2). Human colorectal cancer HCT116 wild type or EP300 knockout (purchased from Cancer Technology) and their GFP-LC3-expressing derivatives were cultured in McCoy medium supplemented with 10% (v/v) fetal bovine serum, 100 mg/L sodium pyruvate, 10 mM HEPES buffer, 100 IU mL^{-1} penicillin G sodium salt, and 100 mg/mL streptomycin sulfate. Murine embryonic fibroblast (MEF) cells and their GFP-LC3-expressing derivatives were cultured in DMEM medium supplemented with 10% (v/v) fetal bovine serum, 100 mg/L sodium pyruvate, 10 mM HEPES buffer, 100 IU mL^{-1} penicillin G sodium salt, and 100 mg/mL streptomycin sulfate and 1 mM non-essential aminoacids. Murine methylcholanthrene induced fibrosarcoma MCA205 cells (class I MHC haplotype H-2b, syngenic for C57BL/6 mice), their GFP-LC3 expressing derivatives and short hairpin RNA (shRNA) transfected cells, as well as murine colorectal cancer CT26 (class I MHC haplotype H-2d, syngenic for Balb/c mice) and non-small cell lung carcinoma TC-1 (class I MHC haplotype H-2b, syngenic for C57BL/6 mice) cell lines, were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 IU mL^{-1} penicillin G sodium salt, 100 μgmL^{-1} streptomycin sulfate, 1 mM sodium pyruvate, 1 mM non-essential amino acids and 1 mM HEPES buffer. All cells were maintained in standard culture conditions (at 37 ºC, under 5% CO2). Cells were seeded in 6-, 96- or 384- wells plates before treatment with 20 mM potassium hydroxycitrate (Sigma Aldrich, St. Louis, MO, USA), 1 mM potassium citrate (Sigma Aldrich, St. Louis, MO, USA), 30 μM SB 204990 (Tocris Bioscience, Bristol, United Kingdom), 30 μM BMS 303141 (Tocris Bioscience, Bristol, United Kingdom), 10 μM simvastatin (Sigma Aldrich, St. Louis, MO, USA), 10 μM mevastatin (Sigma Aldrich, St. Louis, MO, USA), 100 μM mevalonolactone (Sigma Aldrich, St. Louis, MO, USA), 1.25 to 5 mM sodium salicylate (Sigma Aldrich, St. Louis, MO, USA), 3 μM C646 (Sigma Aldrich, St. Louis, MO, USA), 100 nM bafilomycin A1 (LC Laboratories, Woburn, MA, USA), 2 μM mitoxanthrone dhydrochloride (Sigma Aldrich, St. Louis, MO, USA), 10 μM IGF-1 (Prospecbio, East Brunswick, NJ,USA).

Immunoblotting. For immunoblotting, protein extracts obtained by cellular lysis in radioimmunoprecipitation assay (RIPA) buffer were run on 4-12% Bis-Tris acrylamide gels (Invitrogen, Carlsbad, CA, USA) and electrotransferred to 0.2 μM polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA,USA) Non-specific binding sites were saturated by incubating membranes for 1 h in 0.05% Tween 20 (v:v in Tris-buffered saline, TBS) supplemented with 5% non-fat powdered milk (w:v in TBS), followed by an overnight incubation with primary antibodies specific for LC3, phospho-ribosomal protein S6 kinase (Thr421/Ser424), ribosomal protein S6 kinase (Cell Signaling, Danvers, USA), EP300 (Millipore-Chemicon International, Temecula, CA, USA)_or SQSTM/p62 (Abnova). Membranes were cut in order to allow simultaneous detection of different molecular weight proteins. Equal loading was monitored by probing membranes with a glyceraldeyde-3-phosphate dehydrogenase (GAPDH)-specific antibody (mouse monoclonal IgG1 #MAB374, Millipore-Chemicon International, Temecula, CA, USA) or an anti-actin antibody (Abcam, Cambridge, UK). Membranes were developed with suitable horseradish peroxidase conjugates (Southern Biotechnologies, Birmingham, AL, USA), followed by chemiluminescence-based detection with the SuperSignal West Pico® reagent (Thermo Scientific-Pierce, Rockford, IL, USA) and the ImageQuant LAS 4000 software-assisted imager (GE Healthcare, Piscataway, NJ, USA). Quantification was performed by densitometry by means of Image J software.

RNA interference in cell culture. Two different small interfering RNA (siRNA) sequences targeting Acetyl CoA (AcCoA) Carboxylase (Signal Silence AcetylCoA carboxylase, #6224, #6237; Cell Signaling, Danvers, USA) and hydroxymethylglutaryl CoA reductase (#SI00017136, #SI00017150, Qiagen, Valencia, CA, USA) were reversed transfected by means of RNAi MAX transfection reagent.

Microinjection experiments. Human U2OS osteosarcoma cells were cultured overnight on culture dishes before injection. The setup for the microinjection itself was as follows: 10 mM CoA or AcCoA in phosphate buffered saline (PBS) were injected for 0.2 s under an injection pressure of 150 hPa and a compensatory pressure of 9 hPa, using a microinjector (Eppendorf, Hamburg, Germany). Before injection, cells were cultured for 2 h in the presence of 100 nM bafilomycin A1 (Baf A1) in control medium and in presence of 20 mM hydroxycitrate (HC). One hour after injection, cells were fixed with 4 % PFA and analyzed by fluorescent and automated videomicroscopy.

Immunofluorescence. Cells were fixed with 4% PFA for 15 min at room temperature, and permeabilized with 0.1% Triton X-100 for 10 min. For cytoplasmic Acetyl Lysine (Ac-Lys) staining, we proceeded as described in (Pietrocola et al., 2012). Non-specific binding sites were blocked with 5% bovine serum albumin in PBS, followed by incubation with primary antibodies overnight at 4 °C. After 1 h incubation, appropriate Alexa-conjugated Fluorophores (Carlsbad, CA, USA) were used for antigen detection. Ten µM Hoechst 33342 (Molecular Probes-Invitrogen, Eugene, USA) was employed for nuclear counterstaining. Fluorescence wide-field and confocal microscopy assessments were performed on an DM IRE2 microscope (Leica Microsystems, Wetzlar, Germany) equipped with a DC300F camera and with an LSM 510 microscope (Carl Zeiss, Jena, Germany), respectively.

Fluorescent Microscopy. Non-confocal images were acquired with an Axio Observer inverted fluorescence microscope (Carl Zeiss, Jena, Germany). For experiments with human cell lines, a Leica APO 63x NA 1.3 immersion objective was used, whereas for the analysis of GFP-LC3 mice tissue sections, a Leica APO 40x NA 1.15 immersion objective was employed. Zeiss Immersol® immersion oil was used for all microscopic analyses. Images were acquired with a Leica DFC 350 Fxcamera (version 1.8.0) using Leica LAS AF software and processed with Adobe Photoshop (version CS5) software.

Automated microscopy. Human U2OS osteosarcoma cells stably expressing GFP-LC3 were seeded in 96-well or 384-well imaging plates (BD Falcon, Sparks, USA). Twenty-four h before stimulation, cells were treated with the indicated agents for 6 h in absence of lysososomal ATPase inhibitors and for 3 h in presence of Baf A1. Subsequently, cells were fixed with 4% PFA and counterstained with 10 µM Hoechst 33342. Images were acquired using a BD pathway 855 automated microscope (BD Imaging Systems, San Jose, USA) equipped with a 40X objective (Olympus, Center Valley, USA) coupled to a robotized Twister II plate handler (Caliper Life Sciences, Hopkinton, USA). Images were analyzed for the detection of GFP-LC3 puncta in the cytoplasm by means of the BD Attovision software (BD Imaging Systems). Cellular regions of interest, cytoplasm and nucleus, were defined and segmented according to standard procedures. RB 2x2 and Marr-Hildreth algorithms were employed to allow the detection of GFP LC3 puncta. Statistical analyses were conducted using the R (http://www.r-project.org/) and Prism softwares (SanDiego, CA, USA). For quantitative analyses of protein acetylation, cell surfaces were segmented into cytoplasmic and nuclear regions, and the staining intensity of each individual cell was measured for statistical analysis.

Histology and immunohistochemistry. For KRas-induced lung cancer histology, 2 mm sections from at least three different planes were cut and stained with haematoxylin and eosin. Sections were scanned using a Mirax slide scanner and lung/tumor areas were automatically scored by an algorithm programmed and executed using the Definiens software suite and visually analyzed in a double blinded fashion. Immunhistochemical staining was performed by an automatic staining machine (Leica Bond3) or manually processed. Sections were dehydrated and antigenic epitopes were retrieved using a 10-mM citrate buffer and microwaving for 10 min. Specimen were then incubated with rabbit polyclonal anti-Foxp3 (1:100 diluted 13-5773, eBioscience, San Diego, CA, USA), anti-CD3e (1:100 diluted 101442, Santa Cruz, Dallas, TX, USA). Primary antibody staining was detected by peroxidase-conjugated anti-rabbit IgG (1:500 diluted P0448, Dako, Glostrup, Denmark). Positive cells were counted on 20 randomly chosen tumor areas at 400 magnifications in a double blinded fashion. Quantitative analysis was performed using HistoQuest software (TissueGnostics GmbH, Vienna, Austria; http://www.tissuegnostics.com).

Real time *in vivo* imaging of ATP release. Three hundred thousand control or $Atg5^{KD}$ CT26 cells stably expressing a plasma membrane-targeted luciferase protein (pmeLUC) were inoculated subcutaneously (near the thigh) into Balb/C (H-2d) mice, and tumor surface (longest dimension x perpendicular dimension) was routinely monitored using a caliper. When tumor became palpable (about 8 days after injection), mice were divided into four experimental groups receiving (i) *i.p.* 200 µl PBS followed (48 h from the first injection) by 200 µl of PBS; (ii) *i.p.* 100mg/kg HC in 200 µl of PBS followed (48 h from the first injection) by 100mg/kg HC in 200 µl of PBS; (iii) *i.p.* 100mg/kg HC in 200 µl of PBS followed (48 h from the first injection) by 100mg/kg HC in 200 µl of PBS plus *i.p.* 5.17 mg/kg MTX in 200 µl of PBS; and (iv) *i.p.* 200 µl of PBS followed (48 h from the first injection) by *i.p.* 5.17 mg/kg MTX in 200 µl of PBS. Thereafter, mice were anesthetized and imaged with Xenogen - IVIS® Lumina II (Caliper, Hopkinton, MA, USA). D-luciferin (150mg/kg) was administered *i.p.* to mice 15 min before acquisitions, which were performed immediately before and after 48 h from compound injections. The regions of interest were identified around tumor sites and *in vivo* luminescence was identified as photons/s using the Living ImageH software (Caliper, Hopkinton, MA, USA).

Determination of extracellular ATP concentrations. Extracellular ATP levels were measured by the luciferin-based ENLITEN ® ATP Assay (Promega, Charbonnieres, France) kit as indicated by the manufacturer's instructions. ATP-driven chemoluminescence was recorded on a Fluostar multiwell plate lumi nometer (BMG Labtech, Offenburg, Germany).

Tissue sample preparation. Approximately 50 mg of tissues for each condition were weighted and solubilized into 1.5 mL polypropylene microcentrifuge tubes, with a proportional volume of cold lysate buffer (MeOH/Water/Chloroform, 9/1/1, -20°C) (1 mg of tissue in 5 µl of lysate buffer). They were then homogenized two times for 20s at 5.500g using Precellys 24 tissue homogenator (Bertin Technologies, Montigny-le-Bretonneux, France), followed by a centrifugation (10 min at 15.000

rmp 4°C). The upper phase of the supernatant (150 µl) was collected and evaporated in microcentrifuge tubes at 40°C in a pneumatically-assisted concentrator (Techne DB3, Staffordshire, UK). On dried extract, 300 µl of methanol was added and split into two parts of 150 µl: the first one as back-up, and the second one used for the following LC-MS experimentation. After a second evaporation of the aliquots, the LC-MS dried extracts were solubilized with 300 µl of MilliQ water, centrifuged (10 min at 15.000 rmp 4°C) and aliquoted in 3 microcentrifuge tubes (100 µl). Aliquot were transferred in ultra high performance liquid chromatography (UHPLC) vials and injected into UHPLC/MS or kept at -80 °C until injection.

Plasma preparation. A volume of 100 µl of plasma was mixed with a cold solvent mixture (acetonitrile/2-propanol/water, 3/3/2, -20°C), into 1.5 mL polypropylene microcentrifuge tubes, vortexed and centrifugated (10 min at 15.000 rmp 4°C). The supernatant was collected and evaporated in microcentrifuge tubes at 40°C in a pneumatically-assisted concentrator (Techne DB3, Staffordshire, UK). On dried extract, 300µl of methanol were added and 150 µl were used for the following LC-MS experimentation. After a second evaporation of the aliquots, the LC-MS dried extracts were solubilized with 300 µl of MilliQ water, centrifuged (10 min at 15.000 rmp 4°C) and aliquoted in 3 microcentrifuge tubes (100 µl). Aliquots were transferred in UHPLC vials and injected into UHPLC/MS or kept at -80 °C until injection.

Untargeted analysis of intracellular metabolites by UHPLC coupled to a quadrupole- time of flight (QTOF) mass spectrometer. Profiling of intracellular metabolites was performed on a RRLC 1260 system (Agilent Technologies, Waldbronn, Germany) coupled to a QTOF 6520 (Agilent Technologies, Waldbronn, Germany) equipped with an electrospray source operating in both positive and negative mode and full scan mode from 50 to 1000 Da. The gas temperature was set at 350°C with a gas flow of 12 l/min. The capillary voltage was set at 3.5 kV and the fragmenter at 120 V. Two reference masses were used to maintain the mass accuracy during analysis: m/z 121.050873 and m/z 922.009798 in positive mode and m/z 112.985587 and m/z 980.016375 in negative mode. 10 µL of sample were injected on a SB Aq column (100 mm × 2.1 mm particle size 1.8 µm) from Agilent Technologies (Waldbronn, Germany), protected by a guard column XDB-C18 (5 mm × 2.1 mm particle size 1.8µm) and heated at 40°C. The gradient mobile phase consisted of water with 0.2% of acetic acid (A) and acetonitrile (B). The flow rate was set to 0.3 ml/min. Initial condition was 98% phase A and 2% phase B. Molecules are then eluted using a gradient from 2% to 95% phase B in 7 min. The column was washed using 95% mobile phase B for 3 minutes and equilibrated using 2% mobile phase B for 3 min. The autosampler was kept at 4°C.

Targeted analysis of intracellular metabolites by UHPLC coupled to a Triple Quadrupole (QQQ) mass spectrometer. Targeted analysis was performed on an RRLC 1260 system (Agilent Technologies, Waldbronn, Germany) coupled to a Triple Quadrupole 6410 (Agilent Technologies, Waldbronn, Germany) equipped with an electrospray source operating in positive mode. The gas temperature was set at 350°C with a gas flow of 12 l/min. The capillary voltage was set at 3.5 kV. 5 µL of sample were injected on a Column Zorbax Eclipse plus C18 (100 mm x 2.1 mm particle size 1.8 µm) from Agilent technologies (Waldbronn, Germany), protected by a guard column XDB-C18 (5 mm × 2.1 mm particle size 1.8µm) and heated at 40°C. The gradient mobile phase consisted of water with 0.5mM of DBAA (A) and acetonitrile (B). The flow rate was set to 0.2 ml/min, and gradient as follows: initial condition was 90% phase A and 10% phase B, maintained during 4 min. Molecules were then eluted using a gradient from 10% to 95% phase B over 3 min. The column was washed using 95% mobile phase B for 3 minutes and equilibrated using 10% mobile phase B for 3 min. The autosampler was kept at 4°C.

MRM transitions are as follows (all in positive mode):

Compound Name	Precursor Ion	Product Ion	Fragmentor	Collision Energy
Succinyl CoA	868	428	180	25
Succinyl CoA	868	361	180	25
Malonyl CoA	854,1	347,2	45	28
Malonyl CoA	854,1	303,1	45	40
Acetyl CoA	810,1	428	180	20
Acetyl CoA	810,1	303,1	180	28
FAD	786	439	20	20
FAD	786	348	20	20
CoA	768,1	261,2	180	32

NADPH	746	729	200	10
NADPH	746	136	200	40
NADP	744,1	604,1	200	20
NADP	744,1	136	200	50
NADH	666	649	190	10
NADH	666	514	190	20
NAD	664,1	428,1	190	24
NAD	664,1	136	190	50
ATP	508	136	45	40
ADP	428,2	348,1	172	16
ADP	428,2	136	172	28
AMP	348,2	136	99	20
Adenosine	268,1	136	100	16
Adenosine	268,1	119	100	50

Antibodies. For T-cell depletion, monoclonal anti-CD8a (clone 2.43, BioXCell, West Lebanon, NH) and anti-CD4 (clone GK1.5, West Lebanon, NH) antibodies were injected at a dose of 100 µg in the peritoneal cavity at day -2, 0 and +8 post-chemotherapy. Selective T-cell depletion was confirmed by flow cytometry on blood samples. For T-cell neutralization, 100 µg of monoclonal anti-CD73 (clone TY/23, BioXCell, West Lebanon, NH) and 10 µg of monoclonal anti-FR4 (eBioTH6, eBioscience, San Diego, CA, USA) antibodies were injected systemically, respectively through the intraperitoneal route and through the retro-orbital venous sinus at day -2, 0 and +8 post-chemotherapy. For flow cytometry assays, the following monoclonal antibodies were used: anti-CD16/CD32 (clone 2.4G2, BD Biosciences, San José, CA, USA) to block Fc receptors, anti-CD3e-APC or PE-Cy7 (clone 145-2C11, eBioscience, San Diego, CA, USA), anti-CD8a-PE or PerCP-Cy5.5 (clone 53-6.7, BD Biosciences, San José, CA, USA), anti-CD4-PerCP-Cy5.5 (clone RM4-5, eBioscience, San Diego, CA, USA), anti-CD4-APC-Cy7 (clone GK1.5, BioLegend, San Diego, CA, USA), anti-CD25-PE-Cy7 (clone PC61 5.3, eBioscience, San Diego, CA, USA) and anti-ICOS-FITC (clone 7E.1769, eBioscience, San Diego, CA, USA) for detecting cell surface markers and anti-Foxp3-FITC or APC (clone FJK-16s, eBioscience, San Diego, CA, USA) and anti-Ki67-PE (clone B56, BD Biosciences, San José, CA, USA) for intranuclear staining.

Tetramer assay. To detect $CD8^+$ T-cells specific for the TC1 tumor-associated HPV16 E7 viral antigen, we performed a "tetramer" assay as follows. Briefly, tumor-draining lymph nodes were collected and crushed through 100µm cell strainers. After wash, cells were resuspended in complete medium, counted and stained with HPV-16 $E7_{49-57}$ (RAHNIVTF) / $H\text{-}2D^b$ dextramer conjugated with PE (ImmuDEX, Copenhagen, Denmark) during 10 minutes at +4°C. Then, cells were washed and incubated with LIVE/DEAD® Fixable Yellow Dead Cell dye (Invitrogen, Carlsbad, CA, USA) to discriminate viable cells from damaged cells. To detect the cell population of interest by flow cytometry, cells were stained with fluorescent-labeled antibodies targeting T-cell surface markers.

Isolation and phenotyping of tumor-infiltrating lymphocytes. Tumors were dissociated using Miltenyi Biotec mouse tumor dissociation kit (Miltenyi Biotec; Bergisch Gladbach, Germany) according to the manufacturer's protocol. The dissociated bulk tumor cell suspension was resuspended in RPMI-1640, sequentially passed through 70 µm and 30 µm nylon cell strainers (Miltenyi Biotec, Bergisch Gladbach, Germany) and washed twice in PBS. Prior to staining of tumor-infiltrating lymphocytes (TILs) for flow cytometry analysis, samples were incubated with LIVE/DEAD® Fixable Yellow Dead Cell dye (Invitrogen, Carlsbad, CA, USA) to discriminate viable cells from damaged cells. Then, TILs were incubated with antibodies against CD16/CD32 before staining with fluorescent-labeled antibodies targeting T-cell surface markers. Finally, cells were permeabilized and fixed with Foxp3/Transcription Factor Staining Buffer Set (eBioscience, San Diego,

CA, USA) and stained for intracellular Foxp3. Data were acquired using a BD LSRII flow cytometer (BD Biosciences, San José, CA, USA) and analyzed by means of FlowJo v.X PC software (Treestar, OR, USA). Tregs were defined as the $CD3^+CD4^+CD8^-CD25^+Foxp3^+$ T-cell population. Absolute counts of tumor-infiltrating Tregs were obtained taking in account the following parameters: weight of the harvested tumor, total volume of the dissociated tumor cell suspension (cell concentration typically set to 400 mg/ml in PBS), proportion of the whole cell suspension stained (typically 200µl containing 80mg of bulk tumor cell suspension) and proportion of the stained cell suspension ran through the flow cytometer (typically 300 out of 400µl of the stained cell suspension). Cell counts were finally normalized to the mean of absolute Tregs count per mg tumor within the PBS control mice.

Supplemental References

Pietrocola, F., Marino, G., Lissa, D., Vacchelli, E., Malik, S. A., Niso-Santano, M., Zamzami, N., Galluzzi, L., Maiuri, M. C., and Kroemer, G. (2012). Pro-autophagic polyphenols reduce the acetylation of cytoplasmic proteins. Cell Cycle *11*, 3851-3860.