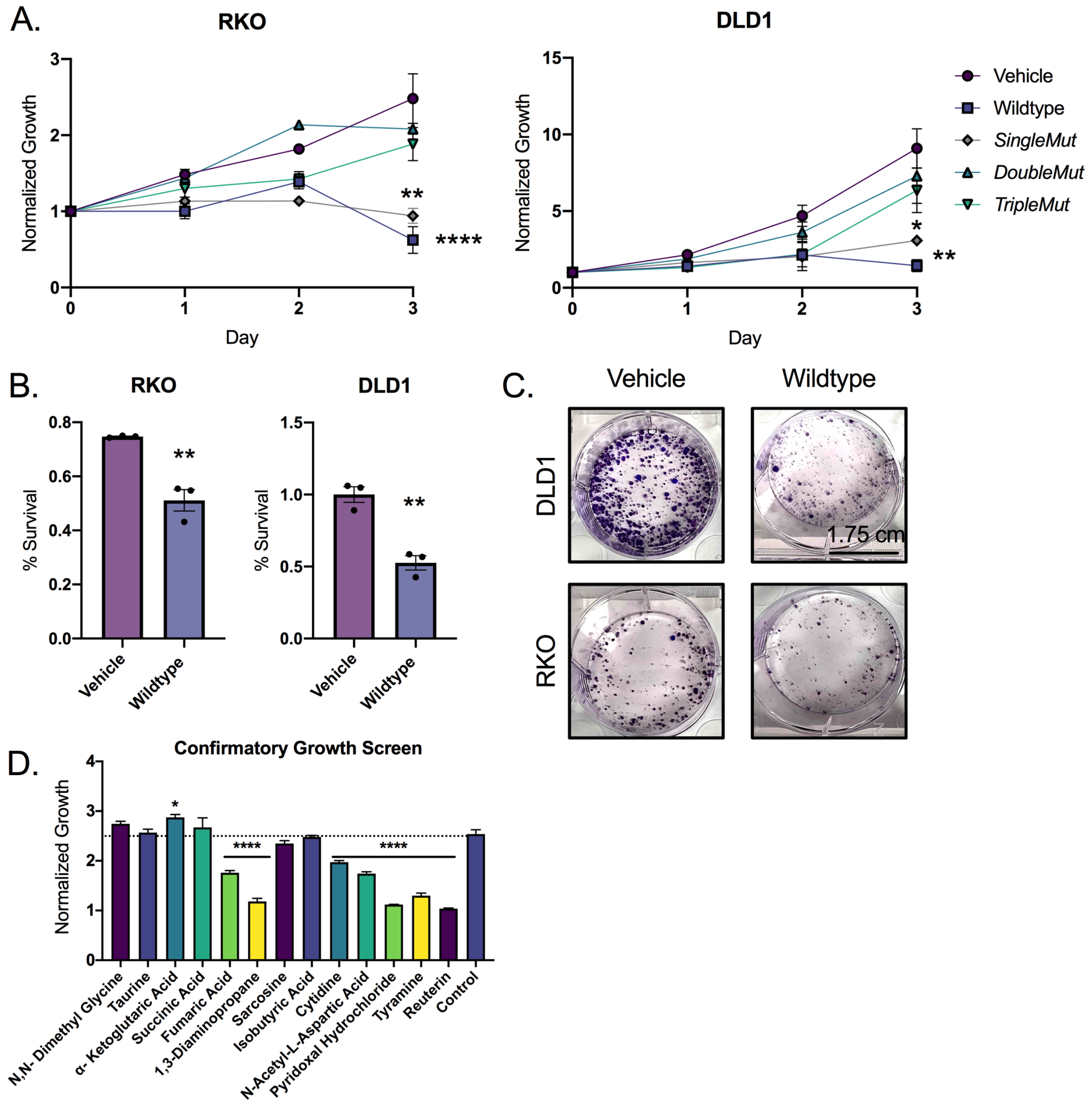
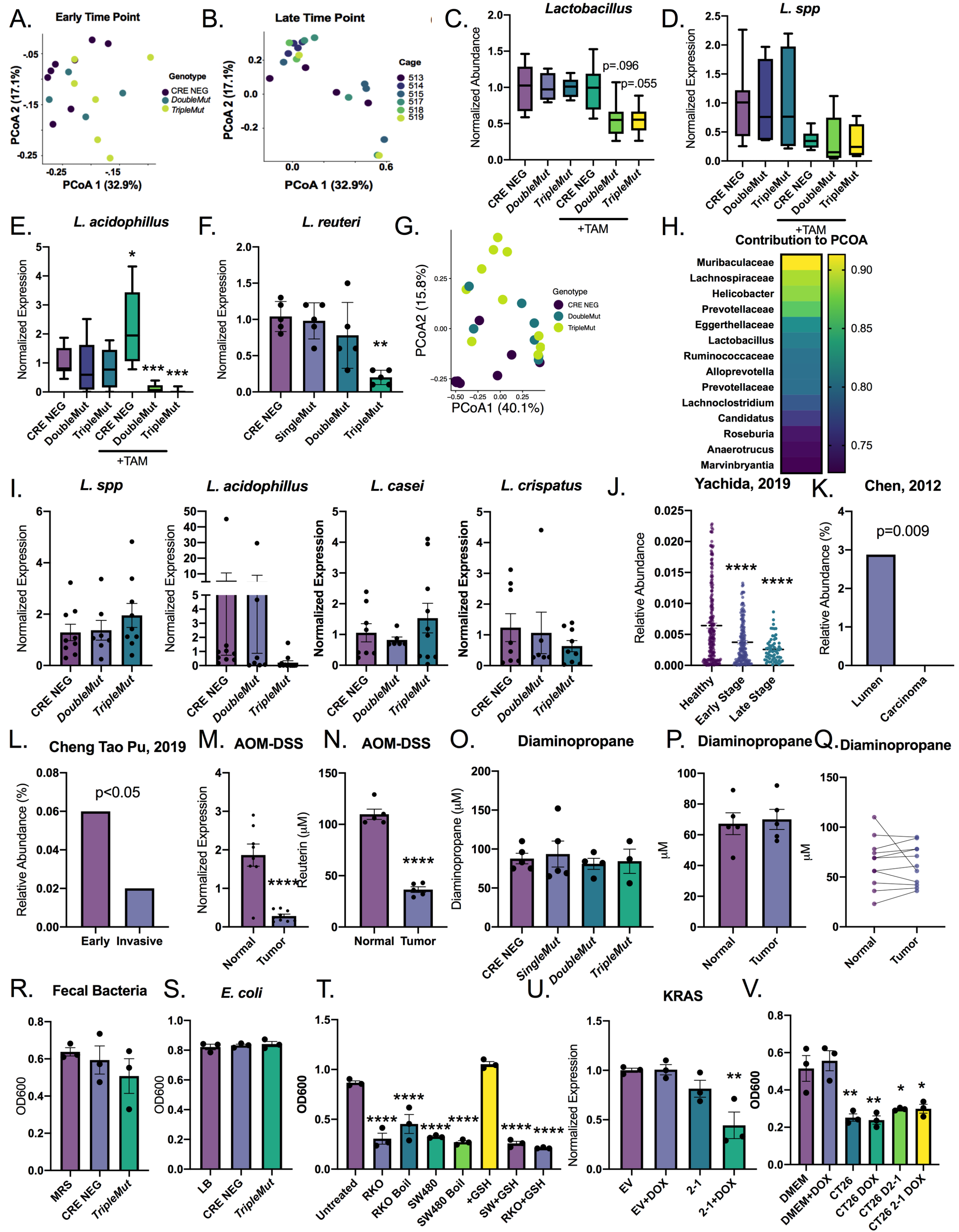


Figure S1: Wild-type fecal metabolites suppress colorectal cancer, related to Figure 1. A) Dried metabolite pellets were resuspended in DMSO by weight and treated at 100X concentration. B) LDH assay of cells treated with 100X fecal metabolites. C) Colony forming assay of cells treated with 100X wildtype fecal metabolites for 14 days. D) Confirmatory growth screen of cells treated with indicated compound at a 500 μM concentration for 72 hours. Statistics were calculated with one-way ANOVA (Panels A and D) or t-test (panel B). *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ unless otherwise indicated. Data is presented as mean +/- the standard error of the mean. All experiments were performed in triplicates at least three times.



A. Early Time Point
PCoA 2 (17.1%)
PCoA 1 (32.9%)
Genotype
CRE NEG
DoubleMut
TripleMut
B. Late Time Point
PCoA 2 (17.1%)
PCoA 1 (32.9%)
Cage
513
514
515
517
518
519
C. Lactobacillus
Normalized Abundance
p=.096
p=.055
CRE NEG
DoubleMut
TripleMut
+TAM
D. L. spp
Normalized Expression
E. L. acidophillus
Normalized Expression
F. L. reuteri
Normalized Expression
SingleMut
G.
PCoA2 (15.8%)
PCoA1 (40.1%)
Genotype
H. Contribution to PCOA
Muribaculaceae
Lachnospiraceae
Helicobacter
Prevotellaceae
Eggerthellaceae
Lactobacillus
Ruminococcaceae
Alloprevotella
Prevotellaceae
Lachnoclostridium
Candidatus
Roseburia
Anaerotrucus
Marvinbryantia
I. L. spp
L. acidophillus
L. casei
L. crispatus
Normalized Expression
J. Yachida, 2019
Relative Abundance
Healthy
Early Stage
Late Stage
K. Chen, 2012
Relative Abundance (%)
p=0.009
Lumen
Carcinoma
L. Cheng Tao Pu, 2019
Relative Abundance (%)
p<0.05
Early
Invasive
M. AOM-DSS
Normalized Expression
Normal
Tumor
N. AOM-DSS
Reuterin (µM)
O. Diaminopropane
Diaminopropane (µM)
P. Diaminopropane
µM
Q. Diaminopropane
µM
R. Fecal Bacteria
OD600
MRS
S. E. coli
OD600
LB
T.
OD600
Untreated
RKO
RKO Boil
SW480
SW480 Boil
+GSH
SW+GSH
RKO+GSH
U. KRAS
Normalized Expression
EV
EV+DOX
2-1
2-1+DOX
V.
OD600
DMEM
DMEM+DOX
CT26
CT26 DOX
CT26 D2-1
CT26 2-1 DOX

Figure S2: *Lactobacillus reuteri* is specifically downregulated in mouse and human colorectal cancer tumors, related to Figure 2. A) PCoA plot of mice before induction with tamoxifen (N=6-8). B) PCoA of mice cage number 14 days after tamoxifen induction. C) Lactobacillus quantification in fecal 16S. Significance is one-way ANOVA. D) qPCR of *L. spp* from fecal 16s DNA. E) qPCR of *L. acidophillus* from fecal 16s DNA. F) Quantification of *L. reuteri* in separate cohort of tumor tissue of indicated mouse model. G) PCoA plot of tumor tissue 16S sequencing. N=(6-8). H) Length of the vector contribution to the PCoA plot by the indicated species. I) Quantification of *Lactobacillus* species by qPCR of 16S tumor tissue DNA. J) Relative abundance of *Lactobacillus* from indicated review of early and late stage tumors. K) Quantification of 16S *Lactobacillus* in the lumen and tumor tissue from indicated paper. L) Quantification of 16S *Lactobacillus* in early and invasive tumors from indicated paper. M)) *L. Reuteri* quantification by qPCR in AOM-DSS mice. N) Reuterin quantification from in AOM-DSS tumors. O) Diaminopropane quantification from tumors in a genetic mouse model. P) Diaminopropane quantification from tumors in an AOM-DSS mouse model. Q) Diaminopropane quantification from paired human normal and tumor tissue. R) Fecal bacterial slurries incubated with indicated fecal extracts. S) *E. coli* incubated with indicated fecal extracts. T) Conditioned media was prepared for 48 hours, then supplemented with 20% MRS broth. Conditioned media was boiled for 10 minutes before MRS addition. 10 mM glutathione methyl ester was added to conditioned media before *L. reuteri* incubation. U) qPCR for KRAS of dox-inducible KRAS knockdown cells V) Conditioned media from Dox inducible KRAS shRNA knockdown cells.. Statistics were calculated with one-way ANOVA (Panels C, D, E, F, I, J, O, R, S, T, U and V) or t-test (panels K, L, M, N, P and Q). *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ unless otherwise indicated. Data is presented as mean +/- the standard error of the mean. 16S experiments were performed a single time with the designated n number, the rest of the experiments were performed at least three times in triplicate.

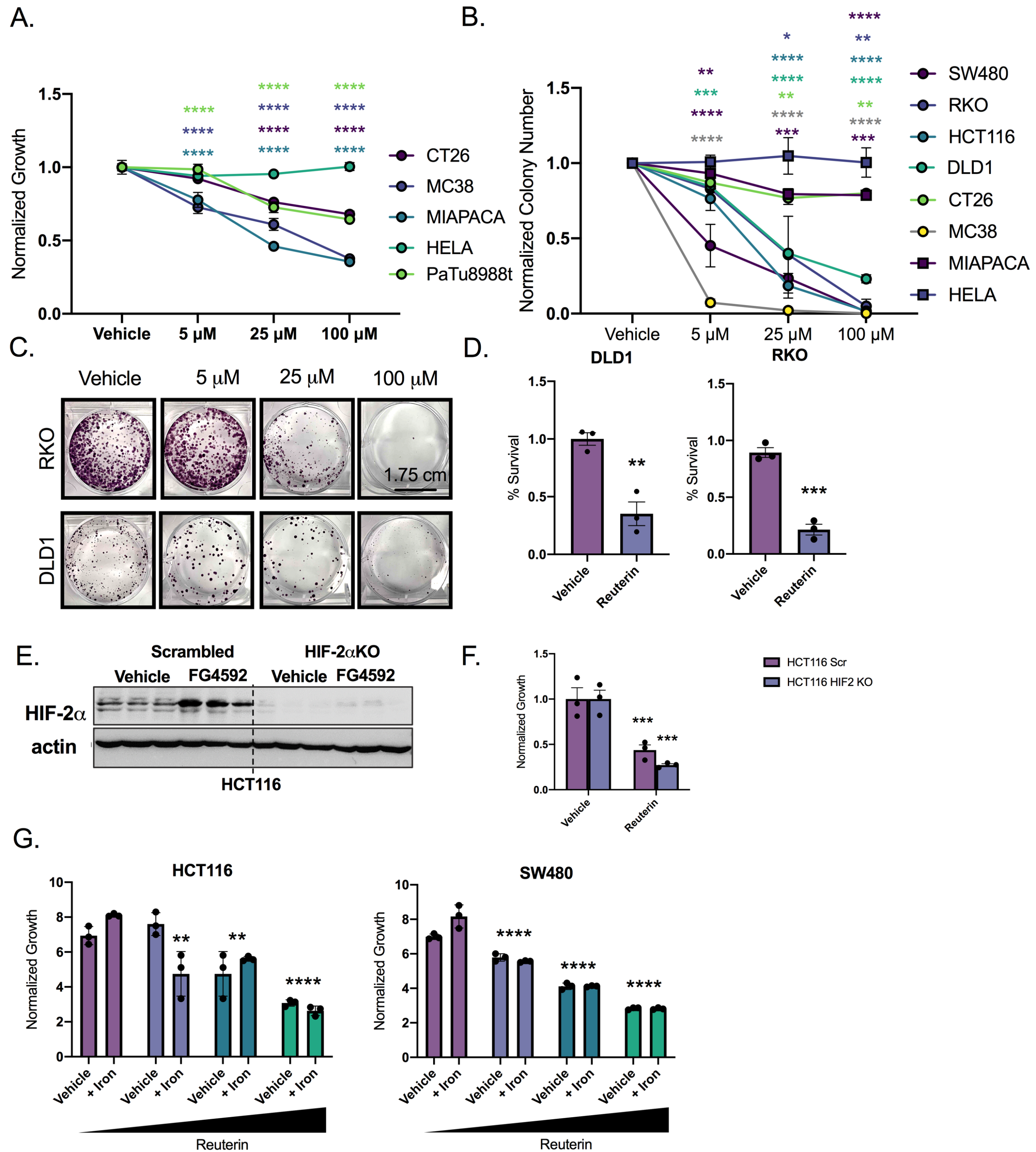


Figure S3: Reuterin growth inhibition is independent of HIF2α or iron, related to Figure 3. A) Dose curve of a panel of cell lines treated with reuterin for 72 hours. B) Quantitation of colony forming assays following reuterin treatment. C) Representative colony forming assay image of reuterin treated cells at 14 days. D) LDH assay of cells treated with 100μM Reuterin for 24 hours. E) Western blot of HIF2α knockout HCT116 cells with and without FG4592. F) Growth assay of scrambled and HIF2α knockout cells treated with 100μM Reuterin for 72 hours. G) Growth assay following treatment with increasing concentrations of reuterin (5-100μM) and

100 μM Ferric Ammonium Citrate (+Iron) for 3 days. Statistics were calculated with one-way ANOVA (Panels A, B, and G) or t-test (panels D and F). *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ unless otherwise indicated. Data is presented as mean +/- the standard error of the mean. All experiments were performed in triplicates at least three times.

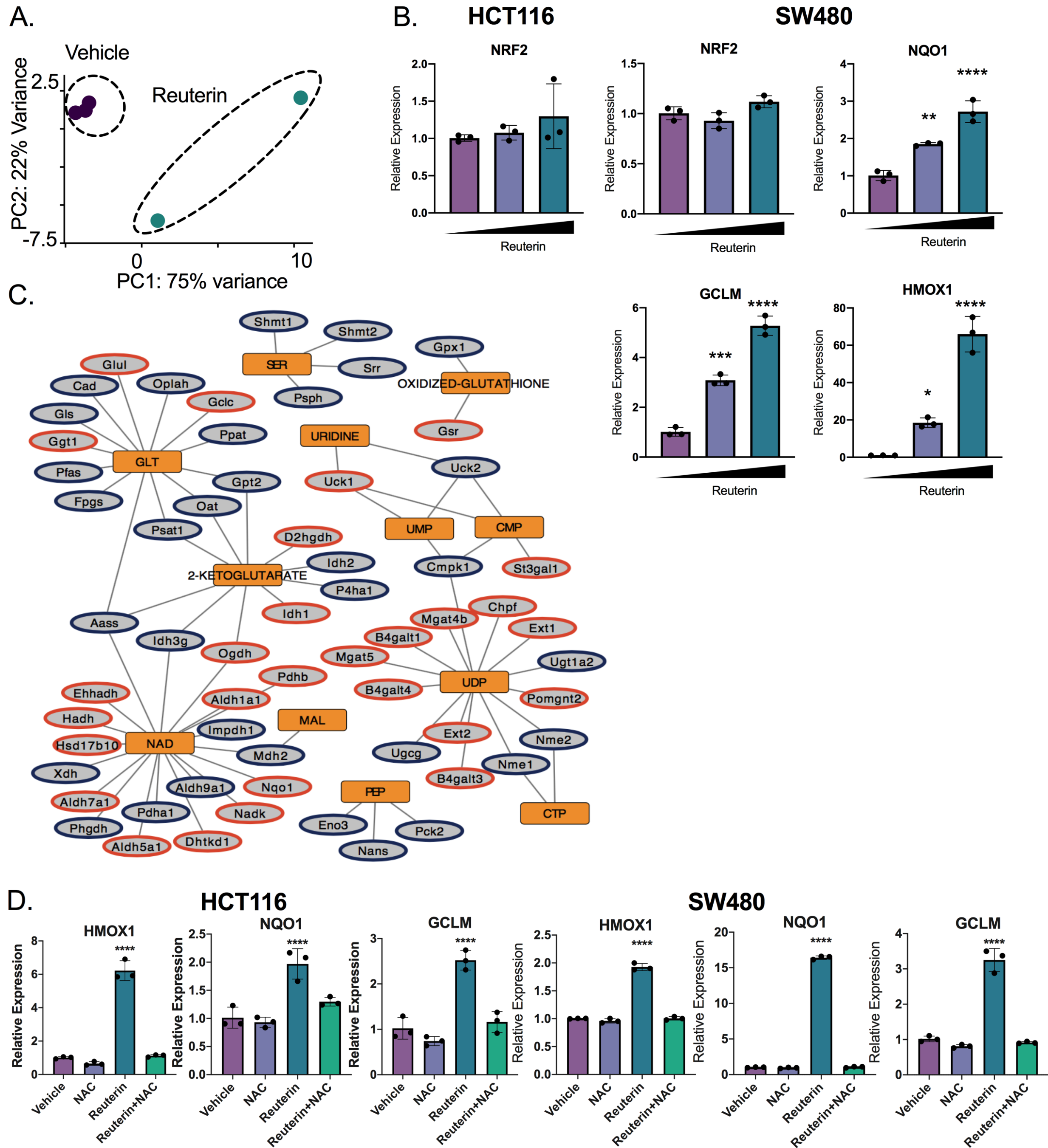
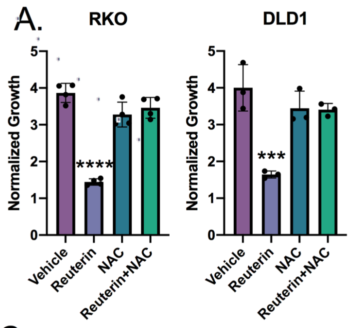
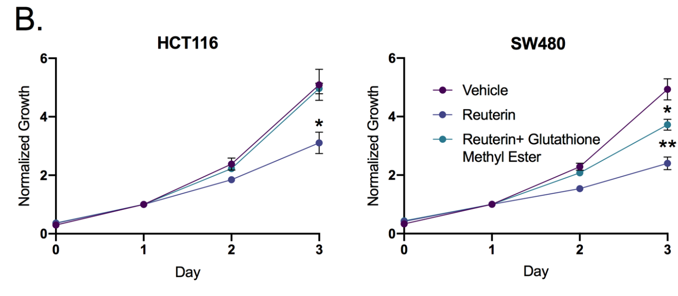


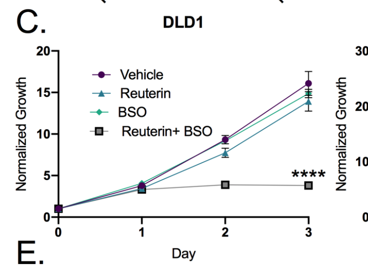
Figure S4: Reuterin potently induces oxidative stress genes, related to Figure 4. A) PCA of Reuterin treated IEC6 cells from RNA-SEQ. B) RT-PCR of Nrf2 target genes after vehicle control, 25µm and 100µm Reuterin treatment for 24 hours. C) Network of metabolites and connected transcripts that are altered in RNA-SEQ. Blue circle is downregulated and red circle is upregulated. D) Cells were pretreated with N-acetyl cysteine 10mM for 24 hours, then treated with 50µm Reuterin for 3 days. Statistics were calculated with one-way ANOVA (Panels B and D). *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ unless otherwise indicated. Data is presented as mean +/- the standard error of the mean. RNA-SEQ was performed in triplicate one time, all other experiments were performed in triplicates at least three times.



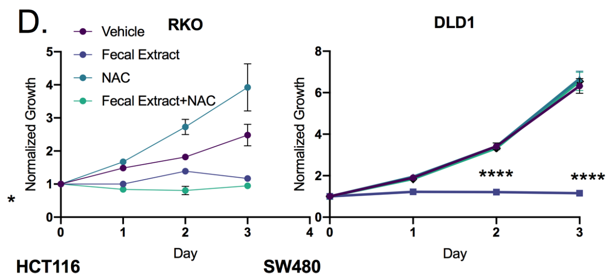
A.
RKO
DLD1
Normalized Growth
****
***
Vehicle
Reuterin
NAC
Reuterin+NAC



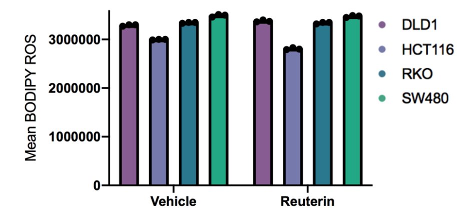
B.
HCT116
SW480
Normalized Growth
Day
Vehicle
Reuterin
Reuterin+ Glutathione Methyl Ester
*
**



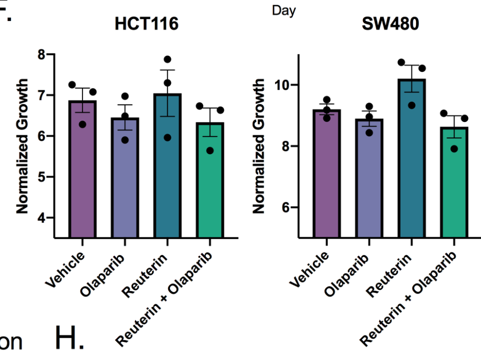
C.
DLD1
RKO
Normalized Growth
Day
Vehicle
Reuterin
BSO
Reuterin+ BSO
****
*



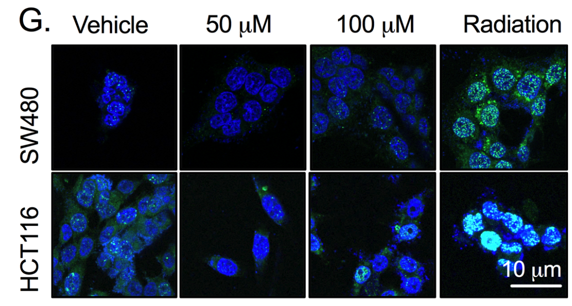
D.
RKO
DLD1
Vehicle
Fecal Extract
NAC
Fecal Extract+NAC
Normalized Growth
Day
****



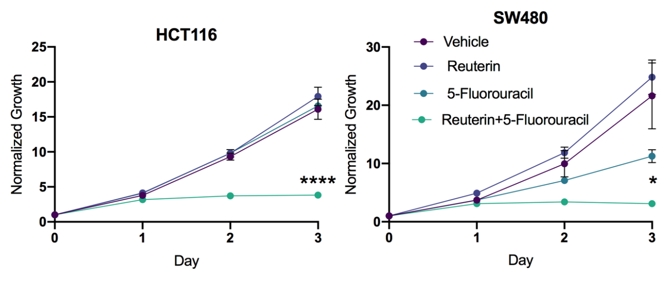
E.
Mean BODIPY ROS
DLD1
HCT116
RKO
SW480
Vehicle
Reuterin



F.
HCT116
SW480
Normalized Growth
Vehicle
Olaparib
Reuterin
Reuterin + Olaparib

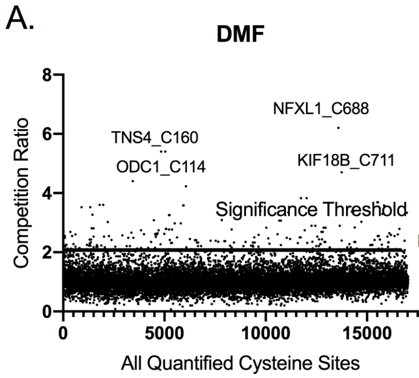


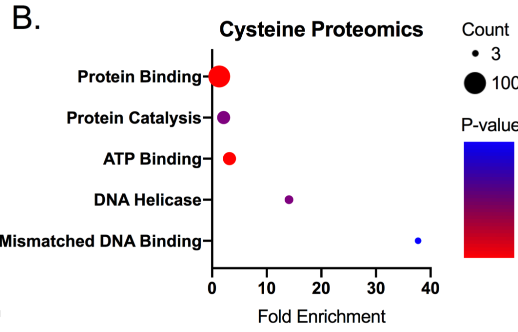
G.
Vehicle
50 μM
100 μM
Radiation
SW480
HCT116
10 μm

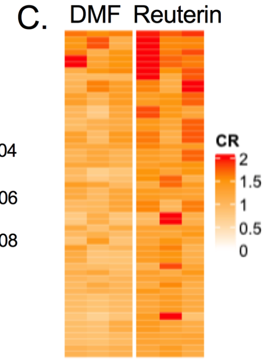


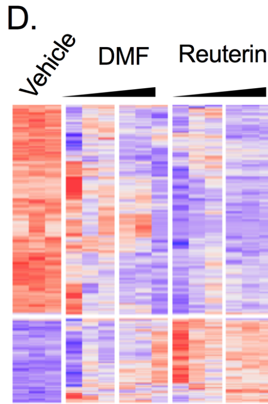
H.
HCT116
SW480
Vehicle
Reuterin
5-Fluorouracil
Reuterin+5-Fluorouracil
Normalized Growth
Day
****
*

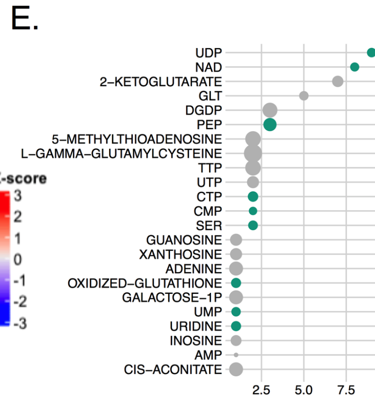
Figure S5: Reuterin does not oxidize lipids or DNA, related to Figure 5. A) Cells were pretreated with NAC for 24 hours, then treated with Reuterin for 72 hours, then analyzed by Cytation imaging software. B) Cells were pretreated with 10 mM Glutathione Methyl Ester for 12 hours, then treated with 50µm Reuterin. C) Cells were cotreated with 10µm Reuterin and 100µm BSO. D) Cells were pretreated with 10mM NAC for 12 hours then treated with 100X Wildtype fecal extract. E) Cells were treated with 100µm Reuterin for 12 hours, then stained with BODIPY dye and assayed by flow cytometry F) Cells were treated with 10µm Reuterin and 1 µm Olaparib and growth was assayed by Cytation at 3 days. G) Cells were treated for 24 hours with 100µm Reuterin or 2 Gy of radiation and imaged for gamma-H2ax foci. H) Cells were treated with 10µm 5-Fluorouracil and 10µm Reuterin. Statistics were calculated with one-way ANOVA (Panels A, B, C, D, E,F, and H). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ unless otherwise indicated. Data is presented as mean +/- the standard error of the mean. All experiments were performed in triplicates at least three times.

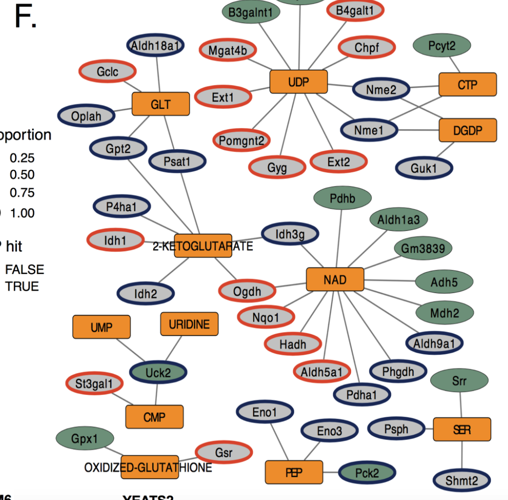

A.
DMF
Competition Ratio
NFXL1_C688
TNS4_C160
ODC1_C114
KIF18B_C711
Significance Threshold
All Quantified Cysteine Sites

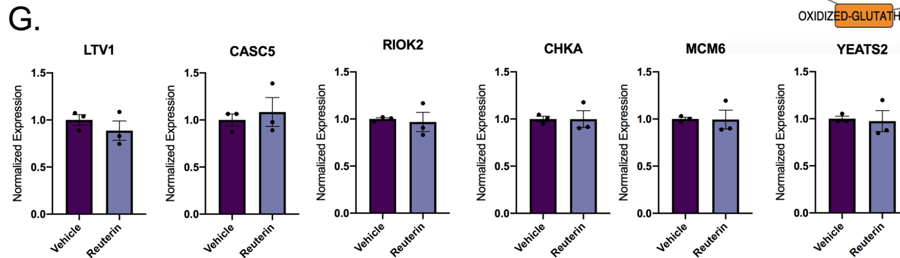

B.
Cysteine Proteomics
Protein Binding
Protein Catalysis
ATP Binding
DNA Helicase
Mismatched DNA Binding
Fold Enrichment
Count
P-value

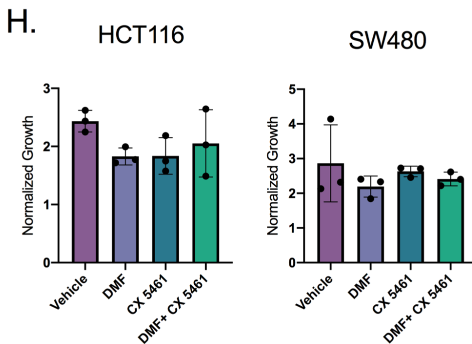

C.
DMF
Reuterin
CR


D.
Vehicle
DMF
Reuterin
Z-score


E.
UDP
NAD
2-KETOGLUTARATE
GLT
DGDP
PEP
5-METHYLTHIOADENOSINE
L-GAMMA-GLUTAMYLCYSTEINE
TTP
UTP
CTP
CMP
SER
GUANOSINE
XANTHOSINE
ADENINE
OXIDIZED-GLUTATHIONE
GALACTOSE-1P
UMP
URIDINE
INOSINE
AMP
CIS-ACONITATE
Proportion
CP hit
FALSE
TRUE


F.
UDP
CTP
DGDP
GLT
2-KETOGLUTARATE
NAD
UMP
URIDINE
CMP
SER
PEP
OXIDIZED-GLUTATHIONE


G.
LTV1
CASC5
RIOK2
CHKA
MCM6
YEATS2
Normalized Expression
Vehicle
Reuterin


H.
HCT116
SW480
Normalized Growth
Vehicle
DMF
CX 5461
DMF+ CX 5461

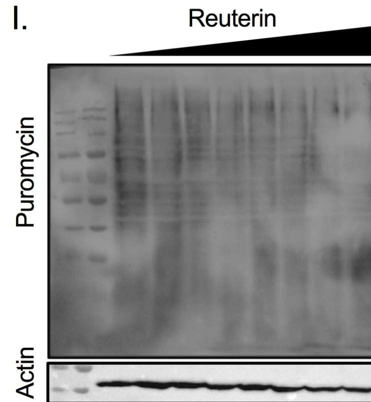

I.
Reuterin
Puromycin
Actin

Figure S6: DMF and Reuterin have distinct mechanisms of growth inhibition, related to Figure 6. A) Competition ratio of average of three separate samples for cysteine sites bound by dimethlyfumarate (DMF). B) KEGG pathway analysis of proteins significantly bound by reuterin. C) Comparison of top cysteine proteomics sites between DMF and reuterin. D) Heat map of proteomics data in DMF and Reuterin treated cells. E) Number of genes in pathways effecting listed metabolite that are differentially expressed in the RNA-SEQ data set. Size of the dot indicates the proportion of linked genes, while green indicates that the gene set contains significant hits from cysteine proteomics. F) Network of metabolites and associated genes. Blue is significantly upregulated and red is significantly downregulated in our RNA-SEQ dataset. Green indicates genes that are significant cysteine proteomics hits. G) QPCR of DEPMAP significant genes in HCT116 cells after 24 hours of treatment with 100 µm reuterin. H) Quantitation of growth on cytation of cells treated with 100 µm dimethylfumarate and 2µm CX-5461. I) Puromycin assay as described in 6I in SW480 cells. Statistics were calculated with one-way ANOVA (Panels H) or t-test (panel G). *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ unless otherwise indicated. Data is presented as mean +/- the standard error of the mean. Cysteine proteomics experiments were performed in triplicate two times. All other experiments were performed in triplicate at least three times.

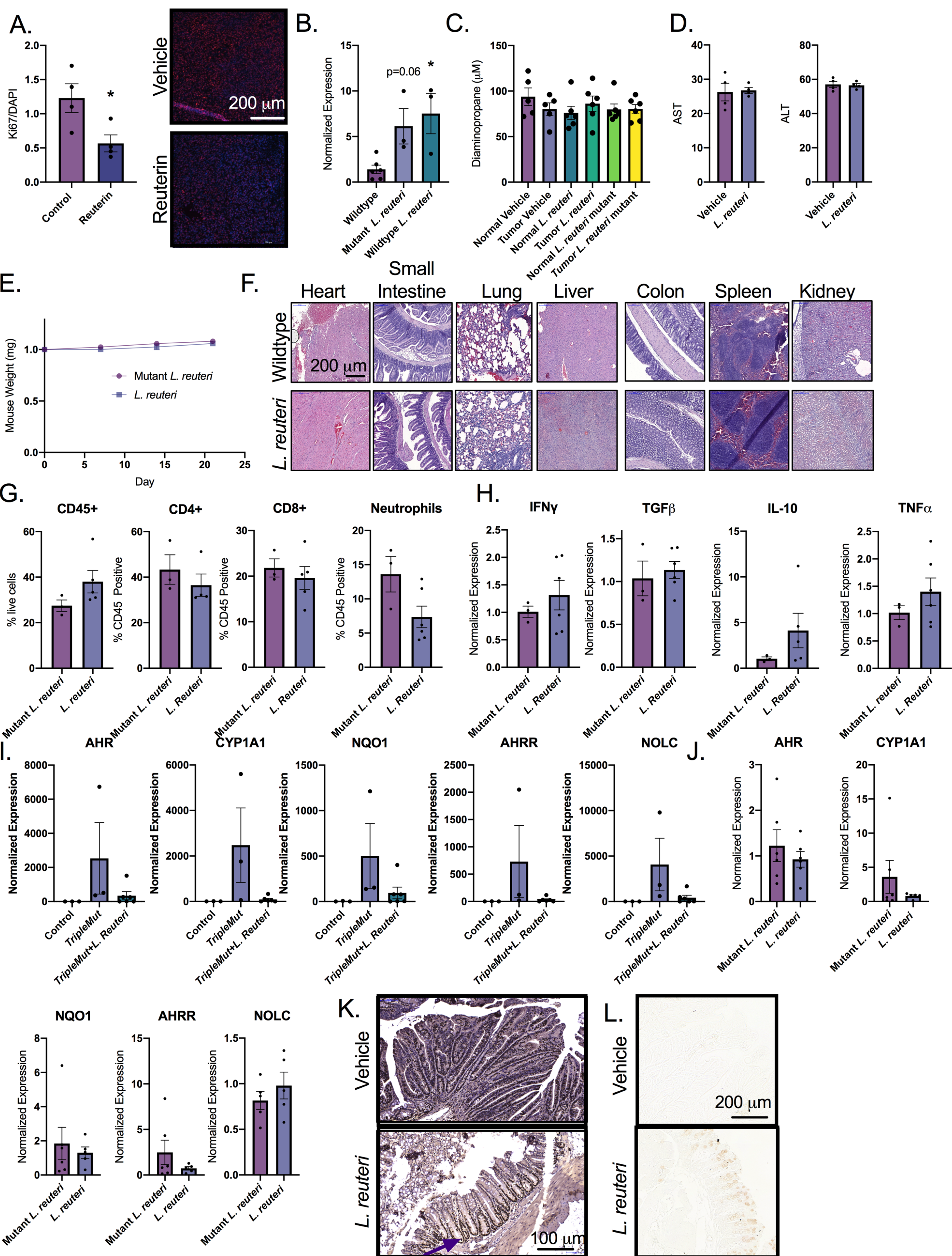


Figure S7: Reuterin reduces proliferation and dysplasia *in vivo*, related to Figure 7. A) Representative Ki67 image and quantification of MC38 reuterin treated syngeneic tumors. (N=3-4). B) qPCR of colons of wildtype mice treated with either mutant or wildtype *L. reuteri* for 7 days. Statistics are one-way ANOVA. C) Quantification of Diaminopropane by mass spectrometry in whole colon from indicated treatment group. D) AST and ALT of wildtype mice treated with *L. reuteri* for 14 days. E) Weight record of mice treated with daily gavage of either wildtype or mutant *L. reuteri.* (N=8) F) Representative H and E of major organs of mice treated with *L. reuteri* for 14 days. (N=6). G) Immune cell flow of induced *TripleMut* mice treated with either mutant or wildtype *L. reuteri* until endpoint. H) qPCR for inflammatory cytokines of the colons of *TripleMut* mice treated with either mutant or wildtype *L. reuteri* until endpoint. I) qPCR of AHR target genes on *TripleMut* mice treated with either mutant of wildtype *L. reuteri* J) qPCR of AHR target genes in MC38 syngeneic tumors gavaged daily with either mutant or wildtype *L. reuteri* daily until end point. K) Representative image of Ki67 staining of PBS treated or *L. reuteri* treated *TripleMut* mice. (N=9). L) Representative image of caspase 3 staining of PBS treated or *L. reuteri* treated *TripleMut* mice. (N=9). Statistics were calculated with one-way ANOVA (Panels B, C, and I) or t-test (panels A, D, G, H, and J). *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ unless otherwise indicated. Data is presented as mean +/- the standard error of the mean. All experiments were performed a single time with the indicated n number.