Dietary fat overcomes the protective activity of thrombospondin-1 signaling in the ApcMin/+ model of colon cancer

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INTRODUCTION

Colon cancer is major public health concern with over 130,000 new cases diagnosed every year and over 50,000 deaths in the United States alone. While many factors including diet and genetics influence colon cancer progression, expression of thrombospondin-1 (TSP1) inversely correlates with colon cancer aggressiveness. TSP1 is a matricellular protein that regulates tissue perfusion, platelet aggregation, angiogenesis, and responses to stress. Spontaneous tumors can be demonstrated in TSP1 null mice only when they are crossed with other strains that are cancer prone such as the MMTV-Neu model or p53 null mice. In several such carcinogenesis models TSP1 expression has been demonstrated to delay premalignant hyperplasia, tumorigenesis, tumor angiogenesis and/or metastasis.

Over 5% of colorectal cancer cases are due to a genetic predisposition, and one frequent abnormality causing predisposition to human colorectal cancer is mutation in the adenomatous polyposis coli (APC) gene. In humans, familial adenomatous polyposis (FAP) is characterized by the onset of multiple adenomas in the colon that can progress to tumors and metastatic forms of colorectal cancer. In mice, heterozygous mutation (ApcMin/+) has generated a clinically relevant model to study colorectal cancer that has provided insights into the onset of sporadic colorectal cancer. These mice develop many of the characteristics of human FAP patients but have some limitations including aggressiveness of the disease and the formation of most adenomas in the small intestine. Still many translational applications have been tested successfully in this model including the use NSAID’s to prevent or treat colorectal cancer. The ApcMin/+ mouse correspondingly is a widely used model for studying these cancers. To monitor the spontaneous colorectal tumor growth, and the survival of mice exposed to a low fat or western diet, we generated a double transgenic animal harboring the ApcMin/+ locus and lacking TSP1 (Thbs1−/−). Previous studies in the ApcMin/+ murine model of colon cancer demonstrated that loss of TSP1 increases tumor multiplicity in the small and large intestines. The absence of TSP1 in this model was correlated with an increase in TUNEL positive nuclei in the polyps lacking TSP1. Therefore, the primary role of TSP1 in carcinogenesis in the ApcMin/+ model was attributed to its role inducing apoptosis. On the other hand, recent studies of mice lacking TSP1 or its receptor CD47 have identified...
important roles in the regulation of lipid and glucose metabolism and the proinflammatory effects of high-fat diets.16–21

In this study, we investigate changes in global liver metabolism associated with the absence of TSP1 in C57BL/6J-ApcMin/J (ApcMin/+ mice), which are highly susceptible to the formation of intestinal adenomas. Liver metabolism responds to changes in metabolites delivered from the intestines and produces metabolites that can influence carcinogenesis in the colon.22,23 As endogenous TSP1 is known to have beneficial effects on colon carcinogenesis in this model in the context of a moderate fat (11%) diet,15 carcinogenic and metabolic effects of low- and high-fat diets were assessed in the context of Thbs1 deletion in this model.

RESULTS

Effects of TSP1 on tumor multiplicity in the ApcMin/+ model are modulated by dietary fat

Consistent with the previous report that loss of endogenous TSP1 increased polyp formation and progression in ApcMin/ mice fed a diet that contained 11% fat,15 ApcMin/+;Thbs1−−/− mice fed a western diet containing 5% fat had decreased survival relative to ApcMin/+ mice fed the same diet (P < 0.02, Figures 1a and b). The former completely succumbed by 200 days, whereas 60% of ApcMin/+ mice remained alive at the same time point (P < 0.03). Feeding a western diet containing 21% fat decreased the survival of ApcMin/+ mice, but the positive effect of endogenous TSP1 on survival was lost in mice fed the high-fat diet (Figure 1b).

Small and large intestines (Figures 1c and d, respectively) were examined under light microscopy to determine whether dietary fat regulates the effects of TSP1 loss on colon carcinogenesis. ApcMin/+;Thbs1−−/− mice that were fed a low-fat diet had a 40% (P < 0.03) increase in adenoma formation in their small intestine (Figures 1c and e) and a 52% (P < 0.02) increase in the large intestine when compared with ApcMin/+ mice fed the same diet (Figures 1d and f). Adenoma formation in the small intestine increased in mice of both genotypes fed a high-fat diet. ApcMin/+ mice fed a high-fat diet had a 60% (P < 0.03) increase in adenoma formation when compared with mice of the same genotype fed a low-fat diet but not when fed a high-fat diet. WT, Thbs1−−/−, ApcMin/+ and ApcMin/Thbs1−−/− mice were fed a low-fat (a) or a high-fat western diet (b) beginning at the time of weaning. (a, b) Survival was measured over time and evaluated using Log-rank (Mantel–Cox test) and Grehan–Breslow Wilcoxon test. Equal numbers of male and female mice were included (N = 14) for each group. (c, d) Small and large intestines were excised at the time of death. (c, d) Small intestine (e) and large intestine (f) lesions were counted under a dissecting microscope and confirmed by H&E staining. N = 4–5, *P < 0.05. (g, h) Representative images of TUNEL staining in small (left) and large intestine of ApcMin/+ and Thbs1−−/−ApcMin/+ mice quantification is presented for small (i) and large intestines (j). N = 6–8 *P < 0.05.

Figure 1. Endogenous thrombospondin-1 limits tumor multiplicity and enhances the survival of ApcMin/+ mice when fed a low-fat diet but not when fed a high-fat diet. WT, Thbs1−−/−, ApcMin/+ and Thbs1−−/−ApcMin/+ mice were fed a low-fat (a) or a high-fat western diet (b) beginning at the time of weaning. (a, b) Survival was measured over time and evaluated using Log-rank (Mantel–Cox test) and Grehan–Breslow Wilcoxon test. Equal numbers of male and female mice were included (N = 14) for each group. (c, d) Small and large intestines were excised at the time of death. (c, d) Small intestine (e) and large intestine (f) lesions were counted under a dissecting microscope and confirmed by H&E staining. N = 4–5, *P < 0.05. (g, h) Representative images of TUNEL staining in small (left) and large intestine of ApcMin/+ and Thbs1−−/−ApcMin/+ mice quantification is presented for small (i) and large intestines (j). N = 6–8 *P < 0.05.

Oncogenesis (2016), 1 – 10
low-fat diet. Moreover, Apc\textsuperscript{Min/−}\textsuperscript{−}, Thbs1\textsuperscript{−/−} mice fed a high-fat diet had 34% (P < 0.05) increased adenoma formation when compared with mice of the same genotype fed a low-fat diet (Figures 1e and f). When fed a high-fat diet, however, lesion formation in the small intestine was not significantly different between mice of these two genotypes (Figure 1f). More relevant to human APC-dependent colon cancers, Apc\textsuperscript{Min/−}\textsuperscript{−}, Thbs1\textsuperscript{−/−} mice fed a high-fat diet had a 48% (P < 0.02) increase in adenoma formation in the large intestine when compared with Apc\textsuperscript{Min/−} mice fed the same diet (Figure 1e). Dietary fat consumption can affect the induction of cell proliferative capacity and death in intestinal tissue.\textsuperscript{24} We assessed cell death in our model by TUNEL staining of tissues (Figures 1g–j). Consumption of a low-fat diet increased TUNEL positive nuclei in Apc\textsuperscript{Min/−} mice to 26% (P < 0.001) when compared with 8% in Apc\textsuperscript{Min/−}, Thbs1\textsuperscript{−/−} (Figures 1g). In large intestines we observed a 40% (P < 0.001) increase in TUNEL positive staining in large intestines of Apc\textsuperscript{Min/−}/Thbs1\textsuperscript{−/−} mice when compared with a 3% increase in Apc\textsuperscript{Min/−}/Thbs1\textsuperscript{−/−} (Figures 1h). However, induction of cell death was inhibited with the consumption of a high-fat diet in all phenotypes (Figures 1i and j). This implies that consumption of high-fat diet inhibits the activation of pro-apoptotic genes, which may explain the reduced tumor numbers observed in the Apc\textsuperscript{Min/−} mice. Therefore, a high-fat diet selectively increases adenoma formation in the small intestine, but the protective effects of endogenous TSP1 in the small intestine decrease when dietary fat levels increase.

TSP1 regulates systemic metabolic responses to a high-fat diet in the Apc\textsuperscript{Min/−} model

Previous studies of Apc\textsuperscript{Min/−}, Thbs1\textsuperscript{−/−} mice focused on local effects of TSP1 on angiogenesis and inflammatory responses in the intestinal microenvironment where adenomas arise.\textsuperscript{15,25,26} However, evidence is accumulating that TSP1 has systemic effects on cell proliferative capacity and death in intestinal tissue.\textsuperscript{24} In addition to the important local role of Apc in intestinal carcinogenesis, systemic changes in metabolism were recently identified that correlate with high-fat diet-induced risk in the Apc\textsuperscript{Min/−} model\textsuperscript{28,29} and patients with adenomatous polyps.\textsuperscript{30} To address whether endogenous TSP1 has systemic effects that could account for its dietary fat-dependent effects on carcinogenesis, we performed a global metabolomics analysis using liver tissue from each genotype of mice fed the low- or high-fat diets. Quantitative data was obtained for 523 named biochemicals, and of these ANOVA analysis indicated that 24% exhibited variation that was mainly attributable to genotype, 37% exhibited variation that was mainly attributable to diet and 31% exhibited variation related to the interaction between diet and genotype (Table 1).

All genotypes showed global variations associated with dietary fat intake, but the absence of TSP1, either in a wild type (WT) Apc\textsuperscript{+/−} background or in the Apc\textsuperscript{Min/−} mice, was associated with larger numbers of metabolites that either increased or decreased when the mice were fed a high-fat diet (Table 2). These increases indicated that the presence of TSP1 globally limits the effects of diet to alter liver metabolism.

To identify specific metabolites that could account for this protective activity of TSP1 we focused on metabolites that were differentially altered by high-fat diet in Apc\textsuperscript{Min/−}}, Thbs1\textsuperscript{−/−} mice when compared with Apc\textsuperscript{Min/−} mice with a WT Thbs1 locus. Of the named metabolites, 124 showed significant differences in expression between these genotypes that were lost or altered when the mice were fed the high-fat diet (Figure 2a). Comparing liver metabolites from Thbs1\textsuperscript{−/−} versus WT mice, we found 86 metabolites that were sensitive to dietary fat, and of these only 20 overlapped with those in the Apc\textsuperscript{Min/−}, Thbs1\textsuperscript{−/−} versus Apc\textsuperscript{Min/−} comparison (Figure 2b). Therefore, some of the metabolic changes can be primarily related to the Thbs1 genotype, but a majority are specific to Thbs1\textsuperscript{−/−} in the Apc\textsuperscript{Min/−} context. The majority of the differentially expressed metabolites that were sensitive to dietary fat in Apc\textsuperscript{Min/−}, Thbs1\textsuperscript{−/−} versus Apc\textsuperscript{Min/−} mice comprised amino acid and lipid metabolites. Eight of the 20 overlap metabolites in Figure 2b were amino-acid metabolites. Most metabolites had higher relative expression in livers of Apc\textsuperscript{Min/−}, Thbs1\textsuperscript{−/−} mice, which were either lost or reversed in livers from mice fed the high-fat diet. This trend is illustrated in Figure 2c for liver amino-acid levels. Levels of 11 of the 20 amino acids were higher in Apc\textsuperscript{Min/−}, Thbs1\textsuperscript{−/−} mice than Apc\textsuperscript{Min/−} mice on the low-fat diet, but of the 20 amino acids were significantly lower in the Apc\textsuperscript{Min/−}, Thbs1\textsuperscript{−/−} mice when fed the high-fat diet. Notably, 10 of the affected amino acids coincide with amino acids that, when quantified in plasma of Apc\textsuperscript{Min/−} mice fed high versus low-fat diet, significantly correlated with adenoma numbers in those mice.\textsuperscript{29} Therefore, we predict that TSP1-dependent changes in liver amino-acid metabolism contribute to the effects of TSP1 to modulate the effect of dietary fat on intestinal carcinogenesis.

Examining two representative amino acids in each of the genotypes reveals that the high-fat diet increased amino-acid levels in WT mice and the two single transgenic strains relative to the respective genotypes fed the low-fat diet (Figure 2d). In contrast, the high-fat diet in the double transgenic Apc\textsuperscript{Min/−}, Thbs1\textsuperscript{−/−} mice broadly decreased levels of the same amino acids. The affected amino acids include the two exclusively ketogenic amino acids, lysine and leucine, as well as three additional amino acids that are partially ketogenic: isoleucine, tryptophan and phenylalanine. This implicates altered mitochondrial metabolism in the broad effects of TSP1 on amino-acid metabolism. The pattern shown for amino acids extends to most of the 36 amino-acid metabolites that exhibited differential modulation by dietary fat, and most of these are known catabolites of the respective amino acids in Figure 2b (Supplementary Table). In some cases the absence of TSP1 may cause elevation of an amino acid by blocking its catabolism. An illustrative example is shown for isoleucine (Figure 2c). The isoleucine catabolite 3-hydroxy-2-ethylpropionate accumulated in liver of both Thbs1\textsuperscript{−/−} mouse strains, but was not affected by the Apc genotype (Figure 2e).

| Table 1. Statistical summary of liver metabolites significantly associated with effects of genotype, diet, or the interaction between diet and genotype based on two-way ANOVA analysis (P < 0.05) |
|---|---|---|---|---|
| Genotype | Diet | Genotype:diet | Main effect | Main effect | Interaction |
| Significantly altered biochemicals | 123 | 195 | 164 |

From analysis of the data set, total of 523 named biochemicals were detected.

| Table 2. Biochemicals quantified in livers from mice of the indicated genotypes that were significantly altered by feeding HFD versus LFD |
|---|---|---|---|
| Total | Increased with HFD | Decreased with HFD |
| WT | 91 | 41 | 50 |
| Thbs1 null | 153 | 83 | 72 |
| Apc\textsuperscript{Min/−} | 97 | 64 | 33 |
| Apc\textsuperscript{Min/−}, Thbs1 null | 177 | 76 | 101 |

Abbreviations: HFD, high-fat diet; LFD, low-fat diet. Data are from ANOVA contrast analysis of the dataset total of 523 named biochemicals detected.
Effects of Thbs1 and ApcMin on lipid metabolism

Consistent with their increased fat consumption, all strains of mice fed the high-fat diet showed the expected hepatic accumulation of various medium-chain and long-chain saturated, monounsaturated and polyunsaturated free fatty acids (Supplementary Table). Elevations in free fatty acids were similar across groups (for example, pentadecanoic acid Figure 3a). Pentadecanoic acid is abundant in butterfat and is a documented metabolic marker for animals fed this diet.31 Increases in select medium-chain free fatty acids were more pronounced in the ApcMin/+:Thbs1−/− samples (Supplementary Table). From the analysis in Figure 2a, 40 of the named lipid metabolites showed differential regulation by high-fat diet in ApcMin/+:Thbs1−/− mice versus ApcMin+/+ mice. Among these, long chain lipids such as oleate generally showed increased levels with high-fat diet that were lost in the ApcMin/+:Thbs1−/− mice (Figure 3b). Conversely, short-chain lipids such as caproate and seven of the detected hydroxy-fatty acids (for example, 2-hydroxypalmitate) showed decreases with the high-fat diet that were absent in the ApcMin/+:Thbs1−/− mice (Figures 3c and d). Therefore, crosstalk between Thbs1 and ApcMin selectively regulates metabolism of a subset of lipids.

Intake of a high-fat diet was associated with hepatic accumulation of fatty acids, which can affect cell energy metabolism and signaling to stimulate carcinogenesis. Both fatty acid oxidation and the observed increases in ketogenic amino acids provide sources of acetyl-CoA. ApcMin/+:Thbs1−/− mice fed the low-fat diet had 1.8-fold elevated levels relative to ApcMin+/+ mice of the ketone body 3-hydroxybutyrate (BHBA, P = 0.02, Figure 3e). This elevation was lost in ApcMin/+:Thbs1−/− mice fed a high-fat diet, representing a significant reduction (P = 0.04, Figure 3e). BHBA is synthesized in the liver from excess acetyl-CoA and utilized by extra hepatic tissues to meet energy demands during periods of starvation. BHBA has been identified as a serum biomarker of colorectal carcinoma and esophageal carcinoma.32,33 Lipid metabolites involved in eicosanoid biosynthesis showed strong dependences on dietary fat as well as TSP1 (Figure 4). The ApcMin mutant is known to increase COX2 expression and consequently prostaglandin PGE2 levels via a CtBP1-dependent mechanism,34–36 and COX2 is known to regulate TSP1 expression,37,38 but no change in PGE2 expression was reported in Thbs1−/− macrophages.39 However, Thbs1−/− liver showed strong elevation in PGE2 levels as well as in P6F2 and 5- and 15-hydroxyeicosatetraenoic acid levels (Figures 4b, c, e and f). This indicates a general inhibitory effect of TSP1 on eicosanoid biosynthesis from n6-polyunsaturated fatty acids. Notably, the high-fat diet suppressed these elevations in Thbs1−/− liver. The high-fat diet was
also inhibitory in the other genotypes, except for Apc\textsuperscript{Min\textasciitilde}:Thbs1\textsuperscript{−−} mice where levels were low in mice fed both diets. Levels of a major nicotinamide catabolite that is produced in liver were consistent with the observed suppression of eicosanoids in the Apc\textsuperscript{Min\textasciitilde}:Thbs1\textsuperscript{−−} mice. 1-Methylnicotinamide is a potent anti-inflammatory and antithrombotic molecule, and its activity was blocked by a COX2 inhibitor.\textsuperscript{40,41} Although expression of its biosynthetic enzyme nicotinamide-N-methyltransferase was induced in some obesity models,\textsuperscript{42} 1-methylnicotinamide levels in WT and Thbs1\textsuperscript{−−} liver were suppressed by high-fat intake and were basally lower in Apc\textsuperscript{Min\textasciitilde} mice (Figure 4g). Remarkably, 1-methylnicotinamide levels were strongly elevated in the Apc\textsuperscript{Min\textasciitilde}:Thbs1\textsuperscript{−−} mouse liver, but it was also suppressed by feeding the high-fat diet. In contrast, further catabolism of 1-methylnicotinamide to N1-methyl-2-pyridone-5-carboxamide was significantly decreased in Apc\textsuperscript{Min\textasciitilde}:Thbs1\textsuperscript{−−} mice relative to Apc\textsuperscript{Min\textasciitilde} mice (Supplementary Table). The proximal target of 1-methylnicotinamide remains unclear, so further study will be required to determine whether it is responsible for the observed suppression of eicosanoids in Apc\textsuperscript{Min\textasciitilde}:Thbs1\textsuperscript{−−} mice.

TSP1 regulates mitochondrial energy metabolism in Apc\textsuperscript{Min\textasciitilde} mice

The global changes in ketogenic amino acid and lipid metabolites are consistent with regulation of mitochondrial metabolism by TSP1. We recently reported that loss of the TSP1 receptor CD47 regulates metabolic flux through the TCA cycle and altered citrate levels by regulation of citrate synthase activity.\textsuperscript{21} Two TCA cycle
intermediates, citrate and isocitrate, showed differential diet-dependent regulation in ApcMin/+ :Thbs1−/− mice versus ApcMin/+ mice (Figures 5a–c). A comparison between ApcMin/+ :Thbs1−/− and Thbs1−/− mice fed the low-fat diet showed more extensive suppression of downstream metabolites in the TCA cycle (Figure 5a). These differences may be attributable to the ApcMin/+ phenotype and were lost when with a high fat intake. These downstream effects of ApcMin/+ on TCA metabolites are consistent with a previous analysis of SW480 colon carcinoma cells expressing mutant Apc that identified succinate, fumarate and malate as Apc-sensitive metabolites. 28 However, no significant differences in TCA metabolites were observed when comparing ApcMin/+ mice with WT mice. In contrast, Thbs1−/− mice showed significant elevations in α-ketoglutarate, succinate, fumarate and malate levels relative to WT mice. High-fat diet decreased these elevations, but α-ketoglutarate and fumarate remained significantly elevated.

Elevation of the oncogenic metabolite 2-hydroxyglutarate (2HG) is associated with several cancers, and 2HG drives progression of these cancers by epigenetic reprogramming.46,47 2HG increases malignant progression by several mechanisms including increasing cancer stem cell characteristics.46 2HG could, therefore, be relevant to the effects of TSP1 in the ApcMin model because tissues in Thbs1−/− mice expressed elevated cMyc and other stem cell characteristics.47 The ApcMin/+ mice showed no changes in 2HG relative to WT mice, but 2HG levels were significantly higher in Thbs1−/− mice on both diets (Figures 5a and d). Levels were also significantly elevated in ApcMin/+ :Thbs1−/− mice versus ApcMin/+ mice fed a low-fat diet, but when fed a high-fat diet the significant elevation was lost. This correlates with the selective protective role of TSP1 in the ApcMin model that was seen only in the low-fat diet. A causative role for 2HG in this effect of TSP1 expression on intestinal carcinogenesis remains to be determined.

**DISCUSSION**

A global metabolic profiling study revealed significant basal, genotype-specific and dietary fat-dependent metabolic differences in liver tissue of WT versus Thbs1−/− and ApcMin/+ mice. Analysis of liver tissue from the double transgenic ApcMin/+ :Thbs1−/− mice revealed additional metabolic changes that could not be
predicted based on those of the two parental transgenic strains. When fed a low-fat diet Apc\textsuperscript{Min/+}:Thbs1\textsuperscript{−/−} mice exhibited a number of metabolic alterations relative to Apc\textsuperscript{Min/+}:Thbs1\textsuperscript{+/−} mice that were lost with high-fat intake. A majority of these involved amino acid, eicosanoid and lipid metabolism. Ketogenic amino acids related to lipid metabolism are indicative of changes in fatty acids. Perturbations in the TCA cycle intermediates and biochemicals related to lipid metabolism are indicative of changes in fatty acid handling and utilization in high-fat diet-fed mice as compared with the other study groups. Cancer cells use amino acids as energy sources or to generate metabolites to fuel density.\textsuperscript{15} Apc\textsuperscript{Min/+}:Thbs1\textsuperscript{−/−} showed increased vascularization in pre-malignant intestinal tissue but no significant differences in vascularization between adenomas and carcinomas between Apc\textsuperscript{Min/+} and Apc\textsuperscript{Min/+}:Thbs1\textsuperscript{−/−}. This also correlated with no differences in expression of VEGF, which led to the conclusion that TSP1 may regulate the initial stages of carcinogenesis when neovascularization to sustain a tumor lesion is low. Correspondingly, once tumors became well established, the effect of TSP1 deletion was decreased. However, our studies indicate that TSP1 may also be involved in later stages of colon carcinogenesis because Apc\textsuperscript{Min/+} animals that were fed a low-fat diet, which can decrease colon cancer formation, showed decreased survival in the absence of TSP1 expression. In the context of a high-fat diet, the absence of TSP1 led to multiple metabolic alterations in livers of 12-week-old mice that could explain late-stage systemic effects of TSP1 on colon cancer progression. Our study indicates that an increase in TCA cycle metabolites could be due to the increased glycolytic flux, which is characteristic of colon cancer.\textsuperscript{53} Consistent with this hypothesis, loss of the TSP1 receptor CD47 altered both glycolytic and TCA cycle flux in a human T-cell line and cd47\textsuperscript{−/−} mice.\textsuperscript{21} However, the present results suggest decreased TCA cycle activity and oxidative phosphorylation. As both β-oxidation and ketogenesis occur in the mitochondria, and medium-chain free fatty acids can freely cross mitochondrial membranes to facilitate β-oxidation, one explanation for the cumulative differential changes observed in Apc\textsuperscript{Min/+}:Thbs1\textsuperscript{−/−} mice fed a high-fat diet may involve mitochondrial dysfunction, resulting in decreased fatty acid oxidation and ketone body formation, along with the accumulation of free fatty acids. Perturbations in the TCA cycle intermediates and biochemicals related to lipid metabolism are indicative of changes in fatty acid handling and utilization in high-fat diet-fed Apc\textsuperscript{Min/+}:Thbs1\textsuperscript{−/−} mice as compared with the other study groups. Cancer cells use amino acids as energy sources or to generate metabolites to fuel

### Figure 5

Thrombospondin-1 and dietary fat regulate TCA cycle metabolites and 2-hydroxyglutarate in Apc\textsuperscript{Min/+} mice. WT, Thbs1\textsuperscript{−/−}, Apc\textsuperscript{Min/+} and Apc\textsuperscript{Min/+}:Thbs1\textsuperscript{−/−} mice were fed a low-fat or a high-fat western diet at the time of weaning. Livers were excised and flash frozen for metabolomics analysis. (a) Citric acid cycle metabolites and ratios for the indicated metabolites in livers from the respective mice fed low- or high-fat diets. Solid red and green cells indicate P < 0.05, and shaded cells indicate 0.05 < P < 0.1. (b) citrate, (c) isocitrate, (d) 2-hydroxyglutarate levels in livers from mice of the indicated genotypes, N = 8.
Atg7 deletion of autophagy-related protein-7 (epithelium and human colon cancer).57,58 In an Autophagy is demonstrated to be activated in murine intestinal body and glucose utilization due to starvation can increase body formation to maintain energy levels. This shift in ketone while leaving peripheral organs to rely on fatty acid and ketone metabolism and the increased anaplerotic substrates in the TCA cycle. In circumstances where fat is the main source of energy, lipids cannot readily enter the TCA cycle via anaplerotic pathways to generate acetyl-CoA.55 In this situation, levels of acetyl-CoA are generated via β-oxidation. Therefore, amino-acid metabolism can serve as a primary source of anaplerotic substrates to sustain the TCA cycle and become depleted during instances of low glucose utilization.55 Another point of regulation of the TCA cycle in our models is indicated by the levels of 3-hydroxybutyrate in Apc<sup>Min+</sup>/Thbs1<sup>−/−</sup> mice. 3-Hydroxybutyrate is generated in the liver from acetyl-CoA during periods of low glucose levels or starvation. This should occur in the context of a low-fat diet that is not deficient in carbohydrate sources, thus indicating a shift in glucose utilization to ketone body formation as a source of energy may be driven by the absence of TSP1 in the Apc<sup>Min+</sup> mice. During starvation, ketone bodies are used as an energy source, and the decreased use of glucose by peripheral organs such as the liver suggest an increased dependence on fat as an energy source. Therefore, any diet containing the following Custom TaqMan SNP Genotyping Assay primers: forward AHD16ZB_F 5′-GGGAAATTTAGACAGTTCTGGCTCT-3′ and reverse AHD16ZB_R 5′-TAAGCAGGGCCATTACCT-3′ and the minor groove-binder probes AHD16ZB_VIC dye-5′-TCTCTCTCTCAACCTT-3′-quencher and AHD16ZB_M TAMRA dye-5′-TCTCTCTCTCAACCTT-3′-quencher. PCR amplification was performed using an Applied Biosystem ViiA 7 Real-Time PCR System: 60°C for 30 s, 95°C for 10 min followed by 40 cycles of 92°C for 15 s and 60°C for 1 min. The Thbs1 locus was genotyped using SNP primers as described.56 Mice were pair-fed a low fat (AIN-76A 5% fat) or a 21% high-fat western diet (AIN76A and D12079B). One set of mice was killed at 12 weeks for metabolic and histologic analyses. Mice were fed a low-fat diet or a high-fat diet until they reached humane endpoints for survival analysis. The two diets contained equal percentages of protein by weight in the form of casein and equal contents of vitamin and mineral supplements. Basal 5% fat in both diets was provided by corn oil, and the high-fat diet was supplemented to 21% with anhydrous milk fat. Carbohydrates provided by cornstarch, maltodextrin and sucrose were adjusted to yield similar caloric content (3902 kcal% in AIN76A and 4686 kcal% in D12079B). One set of mice was killed at 12 weeks for metabolic and histologic analyses. Mice were generated by the absence of TSP1 in the Apc<sup>Min+</sup> mice. This is a less surprising finding as this could indicate that the low levels of amino-acid metabolites are due in part to increased uptake to fuel cell growth. Lower liver levels of ketogenic amino acids and metabolites may reflect higher mitochondrial β-oxidation in mice fed the high-fat diet. Consistent with this hypothesis, amino-acid levels were depleted by high fat intake in drosophila.25 This was attributed to increased levels of urea and uric acid affecting nitrogen metabolism and the increased anaplerotic substrates in the TCA cycle. 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Metabolites were extracted and analyzed by UPLC-MS. The liver tissue metabolomics data were analyzed using Metabolomics Workbench (http://www.metabolomicsworkbench.org/).
frozen and submitted to Metabolon, Inc for extraction and analysis as shown previously. Briefly, the liquid chromatography/mass spectrometry portion of the platform was based on a Waters ACQUITY ultra-performance liquid chromatography and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution.

Histology
Small and large intestines were collected from mice at 12 weeks and at the time of death. Tissues were fixed in 10% buffered formalin and subsequently sent for paraffin embedding. Tissues were stained with hematoxylin and eosin to determine the structure and were examined under light microscopy. TUNEL staining was performed as described previously. Intestinal lesions were counted blindly by a pathologist from the Laboratory of Pathology at the National Cancer Institute in each of the platform was based on a Waters ACQUITY ultra-performance liquid chromatography and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution.

Statistical analysis
Missing values were assumed to be below the level of detection. Biochemicals that were detected in all the samples from one or more groups, but not in samples from other groups were assumed to be near the lower limit of detection in the groups in which they were not detected. In this case, the lowest detected level of these biochemicals was imputed for samples in which that biochemical was not detected. Following log transformation and imputation with minimum observed values for each compound, data were protein normalized by Bradford protein assay, and both an ANOVA contrast and two-way ANOVA with random effects were used to identify biochemicals that differed significantly between experimental groups. An estimate of the false discovery rate (q-value) was calculated to take into account the multiple comparisons that normally occur in metabolomic-based studies. Application of principal component analysis was used to determine separation of study groups (V = 8 WT, TSP1 null, Apoe−/− and N = 7 Apoe−/−,Thbs1−/−). Pathways were assigned for each metabolite, allowing examination of overrepresented pathways. Survival of mice was measured over time and evaluated using log-rank (Mantel-Cox test and Gehan–Breslow–Wilcoxon test, N = 14).

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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