



HHS Public Access

Author manuscript

Gastroenterology. Author manuscript; available in PMC 2026 March 01.

Published in final edited form as:

Gastroenterology. 2025 March ; 168(3): 508–524. doi:10.1053/j.gastro.2024.10.034.

CREEPING FAT-DERIVED FREE FATTY ACIDS INDUCE HYPERPLASIA OF INTESTINAL MUSCULARIS PROPRIA MUSCLE CELLS – A NOVEL LINK BETWEEN FAT AND INTESTINAL STRICTURE FORMATION IN CROHN'S DISEASE

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Study design: WL, RM, THNL, CF, FR; Execution/data collection: WL, RM, THNL, GW, GD, PM, QTN, AM, JHR, RB, VV, IOG, MC, SH, TP, JC, JW, SL, SK, BLC, TQ, SDH, JL, AK, DVW, PS, WJM, IG, CMG; Data compilation and analysis: WL, RM, THNL, GW, GD, PM, AM, JHR, IOIOGG, MC, SDH, TP, JC, JW, SL, SK, DVW, PS, WJM, B.S., IG, CF, JMB, FR; Oversight/advisory: WL, RM, THNL, BLC, TQ, SDH, JL, AK, MC, AII, B.S., CF, PES, FR; Wrote and edited manuscript: All authors; Acquired funding, regulatory approvals: FR

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Abstract

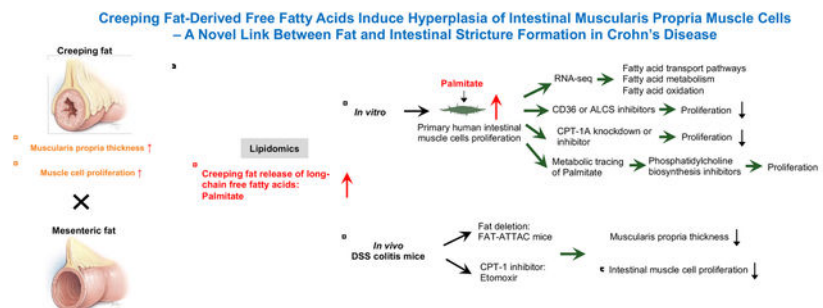
Background: In Crohn's disease (CD) wrapping of mesenteric fat around the bowel wall, so called 'creeping fat', is highly associated with strictures. The strongest contributor to luminal narrowing in strictures is a thickening of the human intestinal muscularis propria (MP). We hence investigated creeping fat derived factors and their effect on mechanisms of human intestinal MP smooth muscle cell (HIMC) hyperplasia.

Methods: Free fatty acids (FFA) in creeping fat or non-creeping mesenteric fat organ cultures were measured via lipidomic mass spectrometry. Primary HIMC were exposed to FFA and cell proliferation was assessed. Intracellular FFA metabolism pathways and reactive oxygen species were functionally evaluated. Muscle thickness was investigated in dextran sodium sulfate (DSS) colitis with small molecule inhibition of FFA transport and a novel fat deletion mouse model.

Results: Subserosal creeping fat is associated with a markedly thickened MP. Experimental deletion of mesenteric fat (FAT-ATTAC mouse) reduced MP thickness. Human creeping fat conditioned medium strongly upregulated HIMC proliferation. Creeping fat released higher amounts of five long-chain FFA, including palmitate. Inhibition of HIMC long-chain FFA metabolism or FFA uptake into mitochondria through carnitine palmitoyltransferase (CPT)-1 reduced the palmitate induced HIMC proliferation. Blockade of conversion of palmitate into phospholipids reduced HIMC proliferation. Prophylactic inhibition of CPT-1 in experimental DSS colitis did not ameliorate inflammation, but reduced MP thickness.

Conclusion: Creeping fat released long-chain FFA induce a selective proliferative response by HIMC. These results point to creeping fat as a novel contributor to stricture formation in CD.

Graphical Abstract



LAY SUMMARY

This work creates an understanding of why the intestinal muscle layer in Crohn's disease strictures thickens and how creeping fat and the muscle layer interact with each other. These findings can be used to identify a potential novel treatment target.

Keywords

Fibrostenosis; Anti-fibrotic therapy; Hypertrophy; Hyperplasia; Lipidomics

INTRODUCTION

The majority of Crohn's disease (CD) patients develop symptomatic intestinal strictures, which comprises a major indication for surgery.¹ Currently there are no specific anti-stricture therapies available despite our increasing understanding of bowel fibrogenesis including intestinal fibroblast activation and extracellular matrix (ECM) deposition secondary to mucosal inflammation.¹⁻³ Recently, intestinal muscularis propria (MP) smooth muscle hyperplasia, but not ECM deposition, was identified as a major contributor to luminal narrowing in stricturing CD.⁴ Targeting intestinal smooth muscle hyperplasia may hence be an important strategy to treat CD associated strictures.

Mesenteric fat wrapping around the inflamed gut, also known as "creeping fat", is pathognomonic of CD.^{5, 6} Evidence suggests that creeping fat is associated with transmural inflammation, MP hyperplasia, excessive ECM deposition, and stricture formation in CD.⁷ The close proximity of creeping fat and the MP makes an interaction between them likely. We previously established a functional interaction between activated human intestinal MP smooth muscle cells (HIMC) and creeping fat as well as non-creeping mesenteric fat.³ HIMC derived fibronectin led to pre-adipocyte migration via integrin mediated mechanisms potentially leading to creeping fat formation.³ However, the interaction of human creeping fat derived mediators contributing to MP hyperplasia has never been investigated.

In the present study, we carefully evaluated factors released by creeping fat and affecting HIMC using primary human cells, lipidomics, RNA sequencing, cell-cell interaction and murine chemical and transgenic models. The results suggest a sequential series of events where subserosal creeping fat releases long-chain free fatty acids (FFA), including palmitate (PA), which induce a proliferative response by HIMC. The PA effect on HIMC proliferation was dependent on uptake into HIMC and the long-chain fatty acid (FA) transporter carnitine palmitoyltransferase (CPT)-1A. PA in HIMC is converted into phospholipids and inhibition of their metabolism reduced PA induced HIMC proliferation. Inhibition of CPT-1 or deletion of fat *in vivo* reduced MP thickness in murine colitis models. These results point to creeping fat as a novel contributor to stricture formation in CD.

MATERIALS AND METHODS

Procurement and histopathology of human intestinal tissues

Full thickness freshly resected intestinal specimens from subjects with CD and controls, comprising ulcerative colitis (UC), diverticular disease and apparently healthy tissue (constipation, healthy margin of resections from colorectal cancer patients; termed NL

for normal) were procured as previously described.^{2, 8–11} CD specimens were classified based on gross anatomy into strictured (CDs) and non-strictured (CDns). Fat was classified into creeping fat or non-creeping mesenteric fat. Creeping fat, as opposed to non-creeping mesenteric fat, was defined by presence of broad-based fat tissue on the anti-mesenteric serosal aspect of the resected intestine on gross examination^{3, 12} or in case the anti-mesenteric serosal side was not available on the resection specimen, creeping fat was defined by its aspect of obliteration or loss of the bowel-mesenteric angle. In our cohort of surgical resection specimens creeping fat was linked to stricturing disease in all cases and no creeping fat without strictures was observed. Creeping fat and non-creeping mesenteric fat can be considered part of the same mesenteric continuum. Hence the term ‘mesenteric fat’, which was used throughout this manuscript for sake of clarity, encompasses also ‘non-creeping mesenteric fat’. CD resections were all derived from the small bowel. This procurement system was validated by histopathologic evaluation performed by a trained IBD pathologist.^{2, 3, 8} Representative histopathologic sections to include the zone over which creeping fat or non-creeping mesenteric fat integrates with the adjoining MP were obtained, formalin fixed and embedded in paraffin.³ 5 µm sections were prepared and slides were stained with hematoxylin and eosin (H&E) and masson trichrome (MT) by the standard methods used in the Anatomic Pathology Department of the Cleveland Clinic.

Measurement of thickness of the muscularis propria

The thickness of the MP on histopathologic specimen was measured as reported previously. Briefly, thickness of MP was determined on HE slides with a DP2-SAL digital camera (Olympus, Tokyo, Japan). Thickness of the MP was measured separately for the inner and outer layer of the MP by observers blinded to the diagnosis and for each layer three measurements were performed and averaged per specimen. Measurements were confirmed by an independent second observer, as reported previously.³

Experimental Dextran Sodium Sulfate (DSS) induced colitis

DSS model with genetic deletion of fat: C57BL/6J FAT-ATTAC mice were generated and kindly provided by Dr. Philipp Scherer, University of Texas Southwestern, Dallas, Texas.¹³ Within two weeks of administration of the dimerizer AP20187 (FK1012 analog) FAT-ATTAC mice show ablation of adipose tissue, without significant secondary effects in other tissues.¹³ Adipocyte progenitors are not affected. This is crucial, as it allows the selective assessment of the effect of fat-derived mediators at the interface of MP and mesenteric fat. 6–8-week-old FAT-ATTAC mice and wildtype (WT) littermates were subjected to administration of dimerizer AP20187 (0.25 mg/kg body weight of mice, intraperitoneal injection, Takara, San Jose, CA, USA) or vehicle (0.4% EtOH, 10% PEG400, 89.6% PBS) twice a week throughout the experiment. After two weeks of injections (four injections), and prior to starting induction of colitis, efficacy of fat deletion was confirmed by measuring whole body fat mass using Echo magnetic resonance tomography (EchoMRI-100H system, EchoMRI LLC, Houston, TX, USA). Colitis was induced by 1.5% DSS (35–50 000 kDa; MP Biomedicals, Santa Ana, CA, USA) in the drinking water for 7 days.

DSS model with administration of the carnitine palmitoyl transferase inhibitor etomoxir: 6–8 week old Balb/c WT mice were subjected to daily administration of etomoxir (60 mg/kg body weight of mice, intraperitoneal injection, MedChemExpress, Monmouth Junction, NJ, USA) or vehicle (10% DMSO, 10% EtOH, 40% PEG400, 40% PBS) the day prior to 3.5% DSS administration (MP Biomedicals, Santa Ana, CA, USA) in the drinking water for 7 days and all throughout the experiment.

The respective DSS doses were chosen after separate dose titration experiments in the above-mentioned mouse strains. Experimental colitis was performed in at least two separate experiments. All mice per group were age-matched, gender matched and were co-housed as derived from the same litter and per genotype to minimize influence from differences in microbial flora composition.¹⁴ Clinical disease activity was determined every other day by measuring body weight loss, stool consistency and presence of occult or overt blood in the stools as previously described.^{2, 8, 11} At the end of the experiment animals were euthanized by CO₂ asphyxiation followed by cervical dislocation. The entire colon was removed, cleaned, and measured from the ileocecal junction to the anus. Histology was performed on paraffin embedded, 3 µm-thick transverse sections stained with hematoxylin and eosin, Masson's trichrome or specific antibodies. Slides were scored by experienced pathologists (I.O.G., S.H.) blinded to the experimental groups using one score separately for inflammation and fibrosis.^{2, 8, 11} The thickness of the MP was calculated on well oriented colon cross sections. Observers blinded to the treatment groups measured the MP thickness underlying fat (mesenteric side) or not underlying fat (anti-mesenteric side) separately with two measurements averaged per MP region using QuPath software.¹⁵

Immunostaining, Organ culture of mesenteric fat and collection of fat conditioned medium can be found in Supplementary Materials.

Lipidomics of non-creeping mesenteric fat and creeping fat conditioned medium

Conditioned medium of non-creeping mesenteric fat and creeping fat were processed for lipidomics and analyzed as previously reported.¹⁶ Briefly, samples were directly transesterified into fatty acid methyl esters by heating samples with sulfuric acid in excess methanol. The resulting fatty acid methyl esters were extracted with petroleum ether, concentrated with a stream of nitrogen and then analyzed by gas chromatography. Fatty acid methyl ester samples were injected into a Shimadzu 2010 gas chromatograph (Shimadzu, Kawasaki City, Japan), separating 30 fatty acids ranging from 12 carbons to 24 carbons. Fatty acids were identified by comparison to authentic standards (NuChek Prep, Elysian MN, USA). Fatty acids were quantified by inclusion of an odd chain internal standard (NuChek Prep Elysian, MN, USA).

Free fatty acid assay quantification kit, Isolation and culture of primary human intestinal cells, Preparation of fatty acids, Quantitative reverse transcriptase polymerase chain reaction, Proliferation Assays, siRNA Transfection of HIMCs, Immunoblotting, Measurement of cellular reactive oxygen species, Measurement of fatty acid esterification and de novo lipogenesis rates in HIMC, Lipidomic profiling of phospholipids in HIMC after exposure to palmitate, additionally used reagents and antibodies, Statistical analysis can be found in Supplementary Materials.

RESULTS

Creeping fat is associated with thickening of the muscularis propria in Crohn's disease

To explore the hypothesis that MP with non-creeping mesenteric fat or creeping fat interactions could lead to thickening of the muscle and intestinal stricture with obstruction, we first investigated the anatomical association of creeping fat and intestinal strictures on gross histopathology (Figure 1A). Creeping fat wrapping around CD small bowel segments was associated with luminal narrowing, which is consistent with previous studies.¹⁷ In fact, all our surgical resections for CD strictures exhibited creeping fat. Upon histopathologic examination (Figure 1B), creeping fat was positioned underneath the serosal layer and, of relevance to this investigation, in direct contact with the MP (Supplementary Figure 1A to further illustrate this spatial relationship). Strikingly, MP interna, externa, or total MP underlying creeping fat in CDs was markedly thicker compared to MP underlying non-creeping mesenteric fat in NL, UC and CDns (Figure 1C; Supplementary Figure 1B; Associated clinical data of tissues used for histopathology in this publication in Supplementary Table 2). A robustly increased number of Ki67 (proliferation marker) positive cells in the MP of CDs tissue underlying creeping fat was noted compared to the MP of CDns, UC and NL tissues underlying non-creeping mesenteric fat (Figure 1D; Additional representative images in Supplementary Figure 1C). A marked amount of Ki67 positive proliferating cells were also positive for the smooth muscle marker desmin suggesting those are muscularis propria smooth muscle cells (Figure 1E&F). This demonstrates an association of the presence of creeping fat with MP proliferation and thickening.

Genetic deletion of fat reduces muscularis propria thickness in DSS colitis.

To assess the principle of fat interacting with the MP, we generated the so-called FAT-ATTAC mouse, in which administration of the dimerizer AP20187 activates caspase-8, deleting adipocytes through apoptosis.¹³ EchoMRI analysis and macroscopic observation confirmed a robust 70% reduction in the body fat mass and reduction in abdominal fat, respectively, in FAT-ATTAC mice compared to baseline within 2 weeks (Figure 2A&B). We next exposed FAT-ATTAC mice or WT controls, pre-treated for two weeks with dimerizer, to DSS to induce colitis for 7 days (Figure 2C). There was no clinically meaningful or significant difference in weight loss, clinical colitis score, clinical colitis subscores or colon length changes in the DSS treated animals throughout the experiment (Figure 2D; Supplementary Figures 2A–C). Histopathologic evaluation of the intestinal segments revealed mesenteric fat tissue atrophy in the dimerizer treated animals (Figure 2E). The degree of inflammation or fibrosis increased in DSS-treated compared to no DSS animals. No difference was observed when comparing DSS WT and DSS FAT-ATTAC dimerizer treated mice (Figure 2F&G), indicating no effect of fat deletion on inflammation or fibrosis. Of relevance, in WT mice with DSS treatment the MP underlying the mesentery thickened. Dimerizer treated FAT-ATTAC mice did not show any increase in MP thickening (Figure 2H, Supplementary Figure 2D). Concordant with these findings, DSS treated WT animals showed an increase in proportion of Ki67⁺ cells in the intestinal MP underlying the mesentery, which was reduced in the FAT-ATTAC dimerizer treated DSS mice (Figure 2I). Most of the Ki67 positive cells also stained positive for desmin, indicating they are smooth

muscle cells (Figure 2J). DSS treated animals showed an increase in colonic inflammatory (*Il1b*, *Il6* and *Tnf*) and extracellular matrix (*Fn1*, *Colla1* and *Col3a1*) gene expression, in the intestinal wall, regardless of FAT-ATTAC or WT background (Figure 2K). This suggests that presence of mesenteric fat is required for MP thickening in experimental colitis, but absence of mesenteric fat does not influence inflammation or fibrosis in the intestinal wall.

Creeping fat-derived free fatty acids induce proliferation of human intestinal muscularis propria cells

To provide a evidence that creeping fat is functionally involved in HIMC proliferation, we generated conditioned medium from human NL, UC & CD non-creeping mesenteric and creeping fat for use in HIMC proliferation (Figure 3A). We purposefully chose this approach to capture actively secreted or released mediators rather than using whole tissue lysates that may contain intracellular contents mixed with secreted or released components. Creeping fat conditioned medium had the strongest effect on HIMC proliferation compared to non-creeping mesenteric fat conditioned medium from CDns, UC or NL (Figure 3B). There was no difference in HIMC proliferation when comparing non-creeping mesenteric fat conditioned medium from CDns, UC or NL. Adipocytes, the major cell type in creeping fat, contain rich lipid droplets, suggesting that lipids, such as free fatty acids (FFA), may be responsible for the observed changes.⁶ Conditioned medium from creeping fat revealed increased concentrations of FFAs compared to CDns, UC and NL non-creeping mesenteric fat conditioned medium (Supplementary Figure 3A). Using unbiased lipidomics, principal component analysis separated creeping fat from CDns, UC and NL non-creeping mesenteric fat conditioned medium (Supplementary Figure 3B). The long-chain FFAs myristic acid (14:0), pentadecanoic (pentadecylic) acid (15:0), palmitic acid (palmitate, 16:0), palmitoleic acid (16:1), oleic acid (18:1), linoleic acid (18:2), γ -linolenic acid (16:3 ω -6) and α -linolenic acid (16:3 ω -3) were significantly elevated in creeping fat compared to controls, with palmitate showing the highest fold increase (Figure 3C; Supplementary Table 3&4). The FFAs myristic acid, pentadecanoic acid, palmitate, palmitoleic acid, and oleic acid markedly induced proliferation of HIMC with palmitate and oleic acid showing the strongest effect (Figure 3D). Due to the robust release of palmitate by creeping fat and its prominent effect on HIMC proliferation we used palmitate for further experiments. Palmitate effect on proliferation was dose dependent with 50 μ M concentration achieving the highest induction of proliferation (Supplementary Figure 3C). On morphological assessment, palmitate increases HIMC cell number, but cell size and shape remained stable (Supplementary Figure 3D). Given the striking effect of long-chain FFAs on HIMC proliferation, we tested a medium chain (decanoate, 10:0) and short chain (butyrate, 4:0) saturated FFA, both of which did not increase HIMC proliferation (Supplementary Figure 3E), indicating that the HIMC response may be confined to long-chain FFA. To enhance rigor, we confirmed the effect of palmitate on HIMC proliferation using two additional methods: EdU incorporation and cell counting (Figure 3E). Increase in proliferation after exposure to palmitate occurred in HIMC derived from NL, UC, CDns and CDs patients in comparable fold changes (Figure 3F). This provides a direct link between FFAs released by creeping fat and HIMC proliferation.

Next generation sequencing of HIMC exposed to FFA of different chain length points to fatty acid transport as a potential mechanism for the palmitate induced HIMC proliferation

To generate hypotheses for later validation of potential pathways for palmitate induced HIMC proliferation, we treated HIMC with palmitate or BSA (control) followed by next generation RNA sequencing (RNA-seq).¹¹ Overall gene expression changes in response to all FFAs were modest. 120 genes were upregulated, and 240 genes were downregulated considering a fold change of 1.1 (Figure 4A). Top 10 upregulated genes were PDK4, HOXA-AS3, MIR570, ANGPTL4, PLIN2, CPT1A, PRKG2, MAP2K4P1, CD200, and LOC652276 (Figure 4A). GO gene set enrichment analysis in palmitate treated cells revealed ion, ATP and DNA binding and cell adhesion among the top ten upregulated pathways and protein targeting to ER, protein targeting to membrane or mitochondrial protein complex among the top ten downregulated pathways (Supplementary Table 5A&B). We next assessed differentially expressed genes and pathways in NL HIMC exposed to palmitate (long-chain FFA), decanoate (medium-chain FFA) or butyrate (short-chain FFA) by RNA-seq and compared them to BSA (control). 265 unique genes were upregulated by palmitate (Figure 4B). GO pathways pointed towards long-chain FFA transport, lipid metabolism and FA oxidation as putative signaling events unique to palmitate, but not decanoate or butyrate treated HIMC (Supplementary Table 5C).

CD36 and long fatty acyl CoA synthetase are critical for palmitate induced HIMC proliferation

Blockade of the FA translocase CD36, an integral membrane transporter importing FFAs into the cell, using sulfo-N-succinimidyl oleate (SSO), markedly decreased the proliferative response of HIMC to palmitate (Figure 4C). Long-chain FA are metabolized via long-chain fatty acid acyl-CoA synthetase (ALCS) into fatty acid acyl-CoA.¹⁸ Inhibition of ALCS in HIMC by Triascin C numerically reduced baseline HIMC proliferation, but entirely blocked the palmitate induced proliferative response (Figure 4D).

CPT-1A is involved in palmitate induced HIMC proliferation

CPT-1A, CPT-1B, CPT-1C and CPT-2 are mitochondrial enzymes with cell type and tissue specific distribution, responsible for transport of long chain FA into the mitochondria.¹⁹ In the intestinal MP, CPT-1A had the highest gene expression, followed by CPT-2, with the lowest expression detected for CPT-1B and CPT-1C (Figure 4E). Immunohistochemistry staining of full thickness bowel resection tissues showed an increase in CPT-1A positive cells in the MP of CDs tissue compared to CDns, UC and NL (Figure 4F). In HIMC derived from different IBD and NL patients, baseline CPT-1A gene expression was the highest, followed by CPT-2 expression, and CPT-1A gene expression was further increased after treatment of HIMC with palmitate, but not decanoate or butyrate (Supplementary Figure 4A). CPT-1A knockdown (Supplementary Figure 4B) did not affect HIMC baseline proliferation rate, but reduced palmitate induced HIMC proliferation almost to baseline levels (Figure 4G). The small molecule CPT-1 inhibitor etomoxir showed comparable effects (Figure 4H). Etomoxir inhibited palmitate induced HIMC proliferation in HIMC derived from all tested phenotypes (NL, UC, CDns and CDs; Supplementary Figure 4C). All other long-chain FFAs secreted by creeping fat in increased amounts that also induced HIMC

proliferation (myristic acid, pentadecanoic acid, palmitoleic acid, and oleic acid) also failed to increase HIMC proliferation in the presence of etomoxir (Figure 4I). Our RNA-seq dataset indicated FA oxidation pathways to be regulated by palmitate and cellular ROS has been linked to increases in cell proliferation²⁰. Palmitate increased total ROS (Figure 4J) as well as mitochondrial ROS (Figure 4K) in HIMC, which was dependent on CPT-1A, as shown by siRNA KD and administration of etomoxir (Supplementary Figure 4D). Scavenging ROS through mito-TEMPO inhibited palmitate induced HIMC proliferation (Figure 4L). Data indicate that long-chain FFA induce HIMC proliferation through FA uptake into mitochondria via CPT-1A and ROS generation.

Metabolic fate tracing of palmitate in HIMC reveals its conversion into phosphatidyl choline

Exogenous FFAs such as palmitate can be an important source of membrane lipids in proliferating cells such as fibroblasts.²¹ Exogenous ³H-labeled palmitate, assessed by one-dimensional thin layer chromatography (TLC), was rapidly converted into phospholipids (PL), triglycerides (TG) and cholesteryl esters (CE), with PL being the major lipid species (Figure 5A). A modest fraction of palmitate was converted into FFAs. ¹⁴C-acetic acid exposure indicated minimal *de novo* synthesis of PL, TG, CE and FA in HIMC, suggesting that the majority of the PL are metabolically derived from exogenous palmitate (Supplementary Figure 5A). Tracing of ³H-palmitate esterification incorporation revealed abundant incorporation into phosphatidyl choline (PC) (Figure 5B). LC tandem mass spectrometry (LC-MS/MS) lipidomic analysis of HIMC exposed to deuterium labelled palmitate, revealed PC and phosphatidylserine (PS) as major glycerophospholipids containing the deuterated label (Figure 5C, top panel). Ranking of all fatty acyl esters across all phospholipids revealed PC and PS subtypes to be the top five highest abundance phospholipids (18:0/16:0, 16:0/16:0, 16:0/18:1, 16:0/16:1; Figure 5C, middle and low panel). Pathway analysis revealed that networks related to PC biosynthesis are enriched by these metabolites. Metabolite set enrichment also revealed phospholipid biosynthesis as the most enriched sets (Supplementary Figure 5B). Interestingly, blockade of the CDP-choline pathway using small molecule inhibitors of glycerol 3-phosphate acyltransferase (GPAT) did not alter the palmitate induced HIMC proliferation (Figure 5D). In contrast, inhibiting the rate limiting step in the classic Kennedy pathway through the choline kinase α inhibitor RSM-932A or CTP:choline-phosphate cytidylyltransferase (CT) inhibitor CT-2584, blunted palmitate induced HIMC proliferation (Figure 5E&F). This indicates that palmitate metabolism through the classic Kennedy pathway is involved in palmitate induced HIMC proliferation (Figure 5G).

Blockade of free fatty acid transport and metabolism in HIMC inhibits proliferation induced by creeping fat conditioned medium

Next, HIMC were directly exposed to creeping fat and CD non-creeping mesenteric fat conditioned medium. Creeping fat conditioned medium again induced proliferation of HIMC and this response was inhibited by blocking CD36, long-chain ACLS or CPT-1A (Figure 6A). Antioxidant mito-TEMPO inhibited creeping fat induced HIMC proliferation (Figure 6B). Upon inhibition of choline kinase α or CTP:choline-phosphate cytidylyltransferase, creeping fat induced HIMC proliferation was also blocked (Figure 6C).

These data support direct relevance of these pathways in the interaction of human creeping fat with the human intestinal MP (Figure 6D).

Administration of CPT-1 inhibitor etomoxir in DSS colitis

We finally sought evidence for the role of CPT-1A in HIMC proliferation *in vivo*. CPT-1 inhibitor etomoxir was administered intraperitoneally daily in a preventive fashion in DSS colitis (Figure 7A). Etomoxir did not affect DSS induced weight loss (Figure 7B), and showed a marginal reduction in clinical score in DSS treated mice (Supplementary Figure 6A; Supplementary Figure 6B). No difference in colon length was observed when comparing DSS vehicle with DSS etomoxir mice (Supplementary Figure 6C). Histologic evaluation for inflammation or fibrosis revealed no difference, when comparing DSS vehicle mice compared to DSS etomoxir mice (Figure 7C–E). Etomoxir reduced thickness in both the MP underlying mesenteric fat and MP not underlying mesenteric fat, when compared to vehicle control in the DSS treated mice (Figure 7F; Supplementary Figure 6D). In DSS mice treated with etomoxir, the percentage of Ki67⁺ cells was reduced compared to DSS mice that only received vehicle control (Figure 7G). The majority of the Ki67⁺ cells also stained positive for desmin (Figure 7H). There was no difference in inflammatory gene expression in DSS mice regardless of etomoxir administration (Figure 7I) indicating that inhibition of CPT-1A reduced muscle proliferation and thickness without affecting inflammation.

DISCUSSION

The role of creeping fat in CD in general and in particular its association with the phenotype of stricturing CD, is just starting to be explored. This is striking as greater than three quarters of bowel segments affected by creeping fat harbor a stricture.²² In our own resection tissues we were in fact not able to identify a single specimen with stricturing disease that was not surrounded by creeping fat and creeping fat ‘creeps’ underneath the serosal layer and directly on to the MP. This feature is unique and can perhaps only be found in epicardial fat in association with atrial fibrillation,²³ or perivascular fat and atherosclerosis.²⁴

While most studies in the field of stricturing disease focus on the excessive deposition of ECM in the intestinal wall as the major culprit for clinical symptoms in patients with stricturing CD¹, Chen and colleagues elegantly described that in fact a thickening of the MP is the strongest contributor to luminal narrowing and hence symptoms of obstruction in our stricture patients.⁴ We confirmed the marked thickening of the MP in patients with stricturing phenotype in our study, but added that once creeping fat was present the MP showed the largest thickness as well as increase in muscle cell proliferation, underscoring the relevance of a potential interaction of the fat with the muscle compartment.

The function of fat is determined by its dynamic and complex composition of distinct cell types, such as immune cells, fibroblasts, among others, with adipocytes, which are rich in lipid droplets, accounting for > 90% of the fat volume.²⁵ Creeping fat contains ample pro-inflammatory cytokines and is metabolically active, for instance secreting large amounts of adipokines. Creeping fat is a major source of TNF.⁶ It was previously unclear if and which type of FFAs are released by creeping fat and how those factors affect adjacent tissue layers. Our novel transgenic loss-of-fat animal model, established that the absence

of mesenteric adipocytes prevents MP thickening in response to inflammation, suggesting that the adipocytes are a requirement for the proliferative MP response seen in humans.⁴ This was confirmed by coculture of human creeping fat conditioned medium with HIMC. Using lipidomics as an unbiased approach, we identified several FFAs, predominantly palmitate, to be released by creeping fat, suggesting active lipolysis, perhaps induced by pro-inflammatory mediators such as TNF within the creeping fat.⁶ This also corroborates recent lipidomics analysis of CD patients and healthy controls, which revealed abnormal lipid metabolism in creeping fat.²⁶ Those FFAs induced a very robust increase in HIMC proliferation, in fact stronger than any previously reported cytokines or growth factors in *in vitro* systems using intestinal fibroblasts or HIMC. Palmitate and other long-chain FFAs inducing proliferation has previously been reported in colorectal cancer cells²⁷ but not in intestinal smooth muscle cells or in smooth muscle cells in general. While our studies suggest a direct effect of FFA on muscle proliferation and that FFA may be required for the MP to thicken, we cannot exclude other factors driving muscle proliferation. In fact, we believe that multiple mediators expressed in the intestinal mucosa, submucosa, MP or fat have the potential to induce smooth muscle hyperplasia and this is part of future investigations.

What remained to be determined were mechanisms of FFA induced proliferation. With guidance of next generation sequencing of HIMC exposed to FFA of different chain length we identified FA transport and metabolism as a putative mechanism. This hypothesis was strengthened by data from other cell types, such as nasopharyngeal carcinoma cells²⁸ and breast cancer cells²⁹, suggesting palmitate signaling driving proliferation through intracellular metabolism, uptake into mitochondria and ultimately undergoing beta oxidation. In fact, we were able to show conclusively with a combination of *in vitro* and *in vivo* studies that palmitate and other long-chain FFA are using CPT-1A to enter mitochondria and increase ROS and that proliferation of HIMC was dependent on those steps.

For cells to divide, an expansion of the cellular membrane is required, which makes a larger pool of phospholipids necessary. As an additional mechanism on how palmitate, and perhaps other FFAs could enhance proliferation, we identified that palmitate is predominantly metabolized into PC. PC is synthesized in cells via the choline metabolism pathways and the first step in the synthesis of PC is the phosphorylation of choline via choline kinase α to form phosphocholine. In the second step, CMP is transferred from CTP to phosphocholine to form CDP-choline by the rate-limiting enzyme CTP:phosphocholine cytidyltransferase (CT).³⁰ In our experimental system, the choline kinase α inhibitor RSM-932A or the CTP:choline-phosphate cytidyltransferase (CT) inhibitor CT-2584 inhibited the palmitate induced HIMC proliferation, indicating that CDP-choline pathway may be involved in palmitate induced HIMC proliferation. Since inhibition of either, the CPT-1A pathway or the choline pathway, were able to completely abrogate the palmitate induced HIMC proliferation, one may speculate that both are required to accomplish the increase cell division needed by HIMC to thicken the MP.

Finally, and most importantly A) when directly exposing HIMC to creeping fat conditioned medium *in vitro* with and without blockade of CPT-1A and choline metabolism inhibitors

we confirmed the identified mechanistic pathways in a human tissue compartment interaction model and B) inhibition of CPT-1A in fact prevents muscle thickening in experimental colitis.

Our study has multiple strengths. To our knowledge this is the first investigation providing a direct functional link of creeping fat with the muscle thickness in stricturing CD. We provide a truly translational approach with primary human cells and tissues as well as a novel transgenic loss-of-function animal model. CPT-1A has not been investigated in relation to intestinal inflammation or strictures. Strikingly the effect of CPT-1A appeared to affect muscle proliferation, but not inflammation or the expression of ECM in our model systems. There are also some limitations to our work. While several animal models for creeping fat have been described^{31, 32} we believe they do not sufficiently reflect the human phenomenon and that a loss-of-function approach was better suited to confirm our hypothesis. To establish the interaction of FA and the MP in experimental colitis, we purposefully chose acute colitis models only, as the MP thickness in this setting is prominent and modifiable. Future studies will examine additional acute and chronic colitis models with prevention and reversal of MP thickening, which was considered out of scope for this already substantial dataset. At that time, we will examine if CPT-1A modulation in fibroblasts in dedicated fibrosis animal models will show an impact on ECM production. The reduction of fat in the FAT-ATTAC mouse is not restricted to the mesenteric fat only but involves fat reduction in the entire mouse body. We cannot exclude that fat loss in other compartments could influence MP thickness in the gut. Due to the limitations of human tissue procurement, we used a combination of paired, strictured and non-strictured, segments derived from the same CD patient as well as individual segments from CD stricture or non-stricture subjects. This is because we required the co-presence of non-creeping mesenteric/creeping fat and muscularis propria and well oriented and present tissue layers in each segment. Due to same restrictions, the number of samples for the lipidomics experiments is modest and hence we put a strong emphasis on a thorough functional validation of the results. We were not able to control our experiments with non-creeping mesenteric fat derived from segments with stricturing CD as all our stricture segments exhibited creeping fat. This is consistent with the literature and identical findings have been reported previously.^{17, 33} It remains to be determined if and how creeping fat influences intestinal fibrosis and inflammation as our work focused on the contribution of FFAs to disease. Future work needs to elucidate further if creeping fat represents a protective response or is an initiator and perpetuator of CD or both.

A significant therapeutic implication of this study is the provision of a mechanistic proof of principle that the effect of creeping fat on the MP can be pharmacologically inhibited. Etomoxir was able to block FFA induced proliferation and was previously tested in clinical development programs for chronic heart failure.³⁴ Concerns about hepatotoxic effects of etomoxir hampered the ultimate use in patients, but more selective, more potent small molecules or local delivery formulations, may make this approach more feasible for future use in patients with CD strictures. Through the work of the Stenosis Therapy and Anti-Stricture Research (STAR) Consortium clinical trials for stricturing disease have now begun ([NCT05843578](https://clinicaltrials.gov/ct2/show/study/NCT05843578) for STENOVA). Clinical trial endpoints have been developed and continue to be refined providing hope to our patients with stricturing CD. Selective inhibition of

muscle thickening in stricturing CD has to date not been tested and we wish our work will put spotlight on this understudied problem.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We thank Dr. Judith Drazba, Dr. John Peterson, Andrele Branicky, and Apryl Helmick from the Lerner Research Institute Microscopy and Image Core, for help in microscopy and image analysis. We thank Dr. Pieter W. Faber (Director, University of Chicago Genomics Facility) for facilitating RNA sequencing. We acknowledge the support of the Departments of Colorectal Surgery and Pathology of the Cleveland Clinic. Tissue samples were provided by the Human Tissue Procurement Facility of the Cleveland Clinic through the services of the Biorepository Core funded by NIH P30 DK097948.

Grant support:

This work was supported by the Helmsley Charitable Trust through the Stenosis Therapy and Anti-Fibrotic Research (STAR) Consortium (No. 3081), the Crohn's and Colitis Foundation (No. 569125), the National Institute of Health (NIDDK R01 123233 & R01 132038 & R01 130227), the National Institute of Health (NIDDK P30 DK097948) to C.F., J.M.B., A.I.I. and F.R., the National Institute of Health (NIDDK K01 131252) to C.M.G., the National Institute of Health (NIAAA P50 AA024333) to J.M.B., Cleveland Clinic through the LabCo program to F.R. and National Science Foundation of China (81970483, 82170537 and 82222010) to R.M. and by the German Research Foundation (CRC-TRR 241-B01 and Z02 (project number 375876048); CRU 5023 (project number 50474582), CRC 1449-B04 and Z02 (project number 431232613); CRC 1340-B06 (project number 372486779)) to B.S..

Conflict of Interest:

I.O.G. receives research support from Celgene Corporation, Morphic Therapeutics, Alimentiv.

For all: IOG receives no direct funds; Cleveland Clinic receives funds on her behalf.

M.C. is consultant to Janssen, Takeda, Abbvie, China Medical System, Ipsen.

S.K. is consultant to Bristol Myers Squibb (BMS).

B.L.C receives the following financial support: Advisory Boards and Consultant for Abbvie, Celgene-Bristol Myers Squibb, Emmes Biopharma Services LLC (DSMB), Lilly, Pfizer, Sublimity Therapeutics, Takeda, TARGET RWE; CME Companies: Cornerstones, Vindico; Speaking: Abbvie; Educational Grant: Pfizer.

T.Q. is a consultant for Abbvie, Celgene/Bristol Meyers Squibb (BMS), Prometheus Biosciences, and Janssen.

S.D.H is a consultant to Takeda.

B.S. is a consultant for AbbVie, Arena, Boehringer Ingelheim, Bristol Myers Squibb, Celgene, Eli Lilly, Endpoint Health, Falk Pharma, Galapagos, Gilead, Janssen, Landos, Pfizer, Prometheus, and Takeda; has received speaker fees from AbbVie, CED Service GmbH, Eli Lilly, Falk Pharma, Ferring, Galapagos, Janssen, Novartis, Pfizer, and Takeda.

C.F. received speaker fees from UCB, Genentech, Sandoz, Janssen and he is consultant for Athos Therapeutics, Inc.

F.R. is consultant to Adiso, Adnovate, Agomab, Allergan, AbbVie, Arena, Astra Zeneca, Bausch & Lomb, Boehringer-Ingelheim, Celgene/BMS, Celltrion, CDISC, Celsius, Cowen, Eugit, Ferring, Galapagos, Galmed, Genentech, Gilead, Gossamer, Granite, Guidepoint, Helmsley, Horizon Therapeutics, Image Analysis Limited, Index Pharma, Landos, Janssen, Koutif, Mestag, Metacrine, Mirum, Mopac, Morphic, Myka Labs, Organovo, Origo, Palisade, Pfizer, Pliant, Prometheus Biosciences, Receptos, RedX, Roche, Samsung, Sanofi, Surmodics, Surrozen, Takeda, Techlab, Teva, Theravance, Thetis, Trix Bio, UCB, Ysios, 89Bio

All other authors declared no conflict of interest.

Data transparency statement:

The transcriptomic datasets will be shared upon request. Please contact the corresponding author for any inquiries. The Cleveland Clinic agrees to use NIH's FDP DUA template to share the data, which can be found at <https://thefdp.org/default/committees/research-compliance/data-stewardship/>. Any other data, analytic methods, and study materials will be made available to other researchers upon reasonable request.

ABBREVIATIONS

α-SMA	α -smooth muscle actin
BSA	Bovine serum albumin
CD	Crohn's disease
CDns	Crohn's disease non-strictured
CDs	Crohn's disease strictured
CF	Creeping fat
CPT	Carnitine palmitoyltransferase
DMEM	Dulbecco's modified Eagle medium
DSS	Dextran sodium sulfate
ECM	Extracellular matrix
FAT-ATTAC	Fat apoptosis through targeted activation of caspase 8
FBS	Fetal bovine serum
FFPE	Formalin-fixed, paraffin-embedded
FPKM	Fragments Per Kilobase of transcript, per Million mapped reads
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HBSS	Hank's balanced salt solution
H&E	Hematoxylin & eosin
HIMC	Human intestinal muscle cell
IBD	Inflammatory bowel diseases
IF	Immunofluorescence
IHC	Immunohistochemistry
LCFA	Long chain fatty acid
LC-MS	Liquid chromatography-mass spectrometry

MCFA	Medium chain fatty acid
MF	Non-creeping mesenteric fat
MP	Muscularis propria
MT	Masson's trichrome
NL	Non-IBD control
qPCR	Quantitative polymerase chain reaction
RPKM	Reads Per Kilobase of transcript, per Million mapped reads
SCFA	Short chain fatty acid
siRNA	Small interfering RNA
UC	Ulcerative colitis

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WHAT YOU NEED TO KNOW

Background and Context

In Crohn's disease (CD), creeping fat is highly associated with strictures. The strongest contributor to luminal narrowing in strictures is thickening of the human intestinal muscularis propria. We investigated creeping fat derived factors and their effect on intestinal muscularis propria smooth muscle cell hyperplasia.

New Findings

Long-chain free fatty acids released by creeping fat induced smooth muscle cell proliferation. This occurs through uptake of fatty acids into mitochondria and their oxidation as well as through lipid metabolism. This is targetable through inhibition of the mitochondrial transporter carnitine palmitoyl transferase 1.

Limitations

Our surgical cohort represents late and already established disease. We are not able to elucidate a link of medications at time of surgery with the findings.

Clinical Research Relevance

An increasing understanding of stricturing CD, a complication for which no selective therapies exist, can lead to development of novel anti-stricture treatments. Blockade of carnitine palmitoyl transferase 1 may be tested in clinical trials for stricturing CD and would for the first time target the muscle thickening and not the deposition of extracellular matrix.

Basic Research Relevance

These results provide a new resource for a better understanding of CD stricture formation. The scientific community and industry can use this information to develop novel therapeutic drug targets. This work for the first time mechanistically links creeping fat with muscle hyperplasia in stricturing CD.

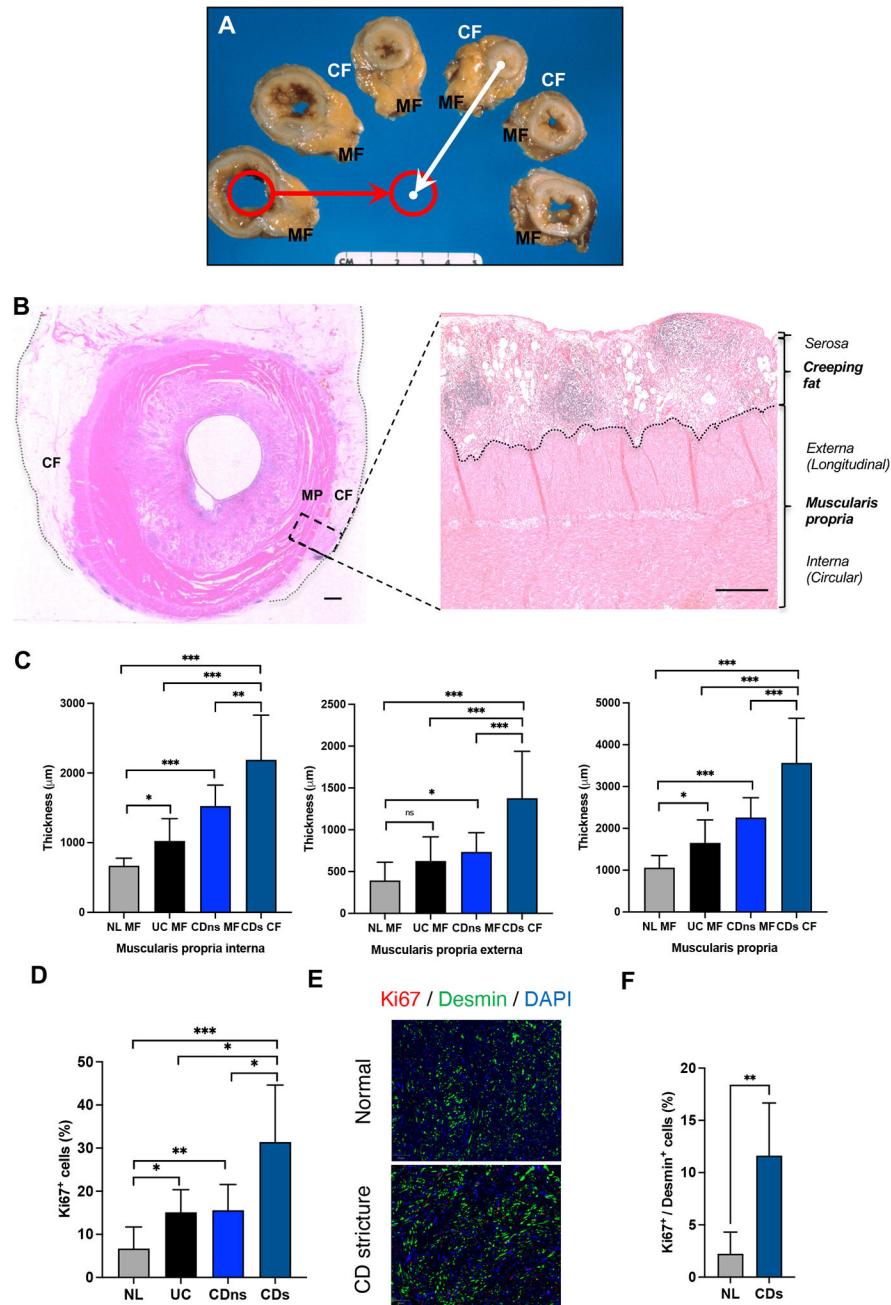


Figure 1. Association between creeping fat, intestinal stricture and muscularis propria thickness. **A.** Gross pathology of intestinal resection tissue rings, cut along the length of an intestinal stricture. Red line indicates the healthy lumen proximal to the stricture. White line indicates the area of maximal luminal narrowing. Creeping fat (CF) is surrounding the intestinal stricture. **B.** Hematoxylin & Eosin (H&E) staining of an intestinal stricture resection. CF (marked by grey dotted line on left) is interposed between the serosa and muscularis propria (MP). The black dotted line marks the zone between CF and MP. Scale bars: 1000µm. **C.** The MP interna, externa and total thickness in CD resection tissues is significantly increased in the MP underlying CF in strictured CD (CDs) compared with the MP underlying non-

creeping mesenteric fat in non-strictured CD (CDns), ulcerative colitis (UC) and normal (NL). N=7–21 per group. **D.** The proportion of Ki67⁺ cells is higher in CDs compared to all other groups. **E.** Representative immunofluorescence stainings of the human MP with a selective antibody to Ki67 (red), desmin (green) and DAPI (blue) in the external MP of NL and CDs are shown. Scale bar: 50µm. **F.** The proportion of Ki67⁺/desmin⁺ is increased in CDs compared to NL with desmin⁺ cells comprising a marked proportion of Ki67⁺ cells. N=6–8 per group. Abbreviations: CF, creeping fat; MF, non-creeping mesenteric fat; MP, muscularis propria; CDs: strictured Crohn's disease stricture; CDns: non-strictured Crohn's disease; UC: Ulcerative colitis; NL: normal tissue; Data are presented as mean ± SEM; ***P<0.001, ** P<0.01, * P<0.05

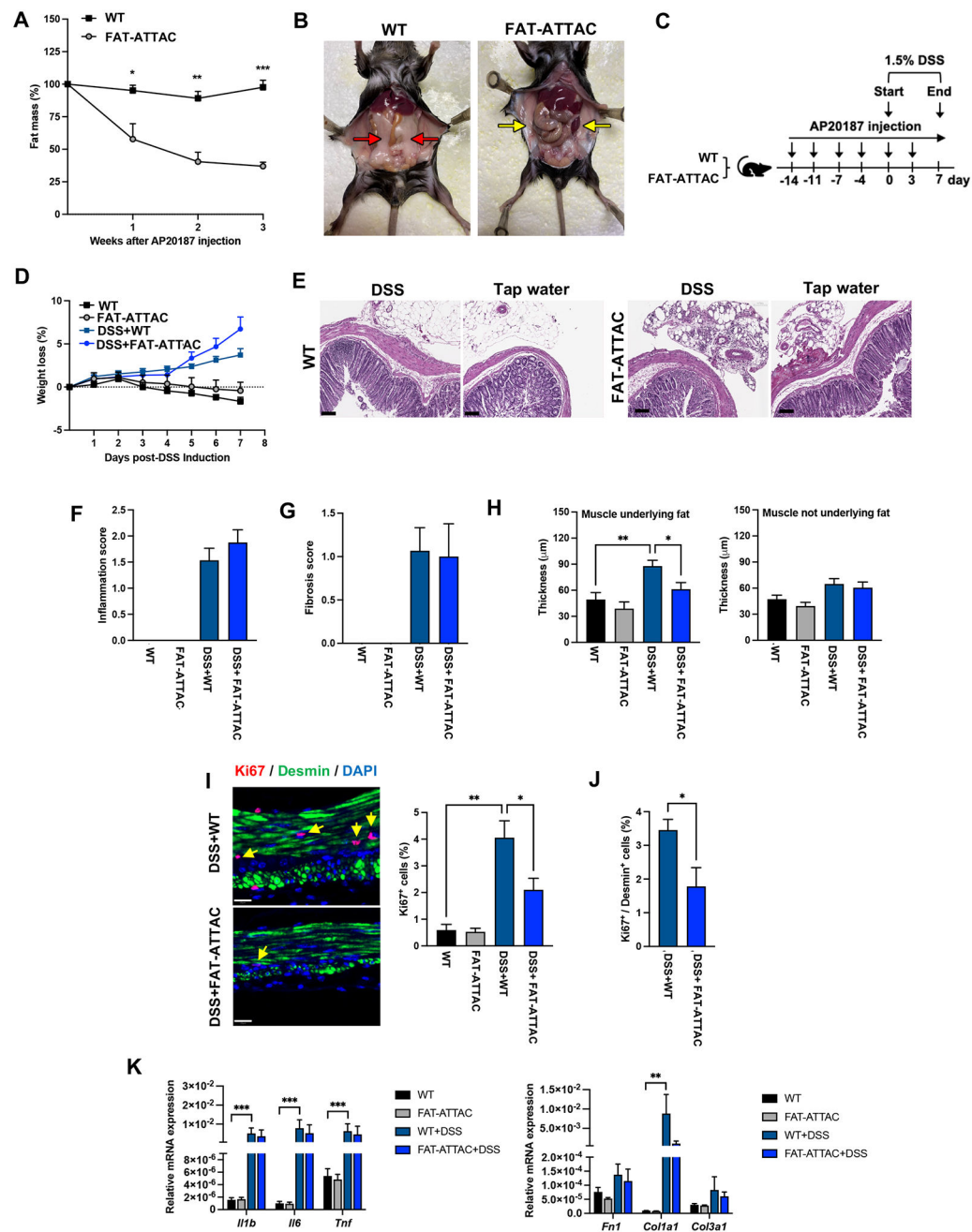


Figure 2. Inducible genetic deletion of fat reduces muscularis propria thickness in DSS colitis.

A. FAT-ATTAC mice or WT mice were subjected to administration of the dimerizer AP20187 twice weekly. Deletion of fat was determined measuring whole body fat mass via Echo magnetic resonance tomography with maximal fat deletion observed starting at week 2. **B.** Representative images of mice euthanized two weeks after fat deletion are shown. Presence of abdominal fat in WT treated animals is marked with red arrows. Absence of fat in FAT-ATTAC dimerizer treated animals is marked with yellow arrow. **C.** Experimental diagram for the DSS colitis model. FAT-ATTAC or WT mice were subjected to administration of the dimerizer AP20187 or vehicle twice weekly starting two weeks

prior to 1.5% DSS administration and all throughout the experiment (N=7–15 per group). **D.** The severity of DSS induced colitis was evaluated by measuring the body weight loss. **E.** Colonic sections were fixed and stained with hematoxylin and eosin (H&E) for histological examination. Representative images are shown. Scale bar: 100µm. **F.** Colonic inflammation score was determined using H&E sections scored by an IBD pathologist blinded to the experimental groups. **G.** The severity of fibrosis was evaluated by an IBD pathologist blinded to the experimental groups using Masson trichrome stains. **H.** Thickness of the colonic muscularis propria (MP) was measured separately for MP underlying mesenteric fat and the MP not underlying mesenteric fat. Measurements were performed by QuPath software. **I.** Immunofluorescence for determination of percent cells positive for proliferation marker Ki67 in the colonic MP underlying mesenteric fat with a selective antibody to Ki67 (red), desmin (green) and DAPI (blue). Scale bar: 20µm. Representative Ki67⁺ cells are marked with yellow arrows. Quantification in bar graph. **J.** Percent positive muscle cells (desmin) that also express the proliferation marker Ki67 indicating the majority of Ki67⁺ cells being muscularis propria smooth muscle cells (N=5). **K.** Gene expression of inflammation and fibrosis genes in intestinal tissues relative to 18S ribosomal RNA (N=6–12). Abbreviations: FAT-ATTAC: fat apoptosis through targeted activation of caspase 8; AP20187: a FK1012 analog; DSS: Dextran sodium sulfate. Data are presented as mean ± SEM; ***P<0.001; **P<0.01; *P<0.05.

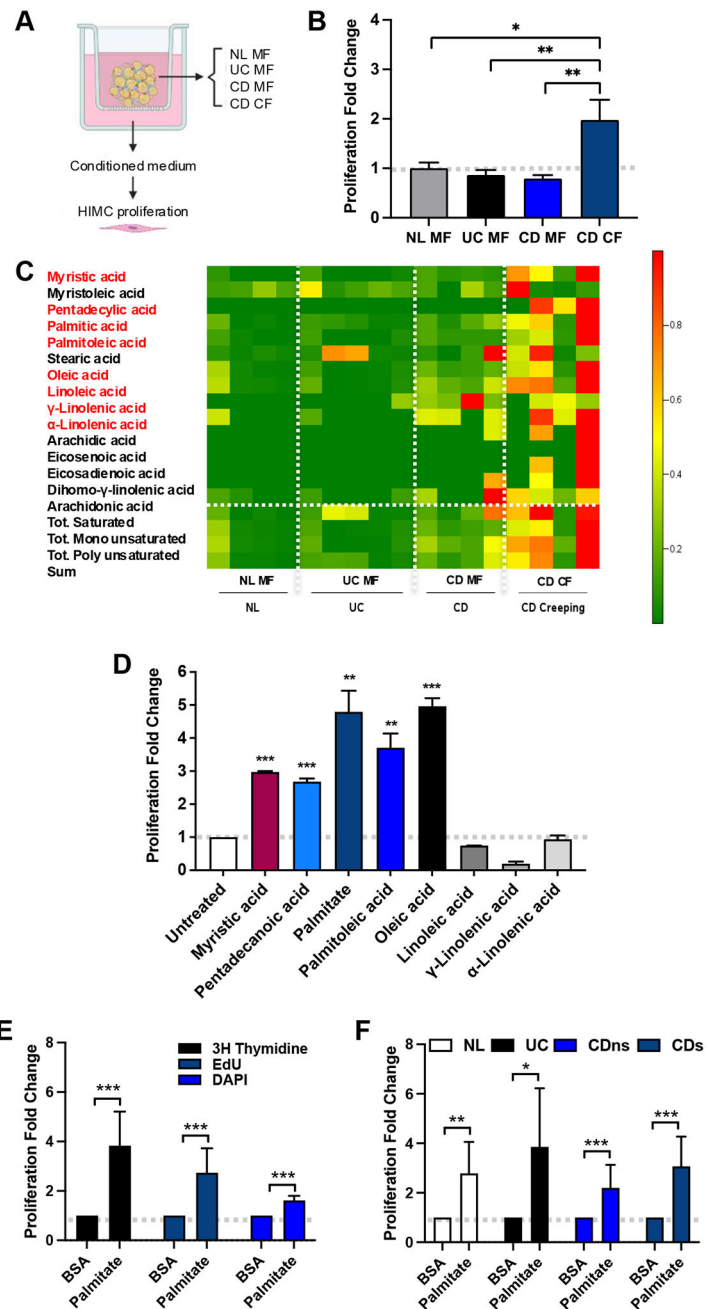


Figure 3. Creeping fat derived fatty acids induce proliferation of primary human intestinal muscularis propria smooth muscle cells.

A. Experimental diagram for generation of conditioned medium by culture of non-creeping mesenteric fat (MF) from normal (NL), ulcerative colitis (UC), Crohn's disease (CD) and CD creeping fat (CF), their collection and use in a human intestinal muscularis propria muscle cells (HIMC) proliferation assay for 48h. **B.** CD CF conditioned medium induces muscle cell proliferation. Proliferation measured using 3H-thymidine. N=4–6 per group. **C.** Heatmap of lipidomic analysis of CF and MF derived from NL, UC, CDns and CDs. FFA significantly elevated in CD CF are colored in red font. N=4–5 per group. **D.** HIMC proliferation after exposure to 50 μ M of different FFA for 48h as measured by 3H-thymidine

proliferation assay. N=3 biologic replicates per group, except data for linoleic acid and oleic acid are technical replicates shown from one HIMC line. **E.** HIMC proliferation after exposure to 50 μ M palmitate for 48h as measured by 3H-thymidine assay, EdU assay and DAPI nuclear count. N=25–28 per group. **F.** Proliferation of HIMC derived from NL, UC, CDns, CDs with or without 50 μ M palmitate for 48h as measured by EdU assay. N=5–10 per group. Abbreviations: CF: Creeping fat; MF: Non-creeping mesenteric fat; CDs: Crohn's disease stricture; CDns: non-strictured Crohn's disease; UC: Ulcerative colitis; NL: normal tissue; Tot.: total; LCFA: Long chain fatty acid; MCFA: Medium chain fatty acid; SCFA: Short chain fatty acid; HIMC: Human intestinal muscularis propria muscle cells. Data are presented as mean \pm SEM; ***P<0.001; **P<0.01; *P<0.05.

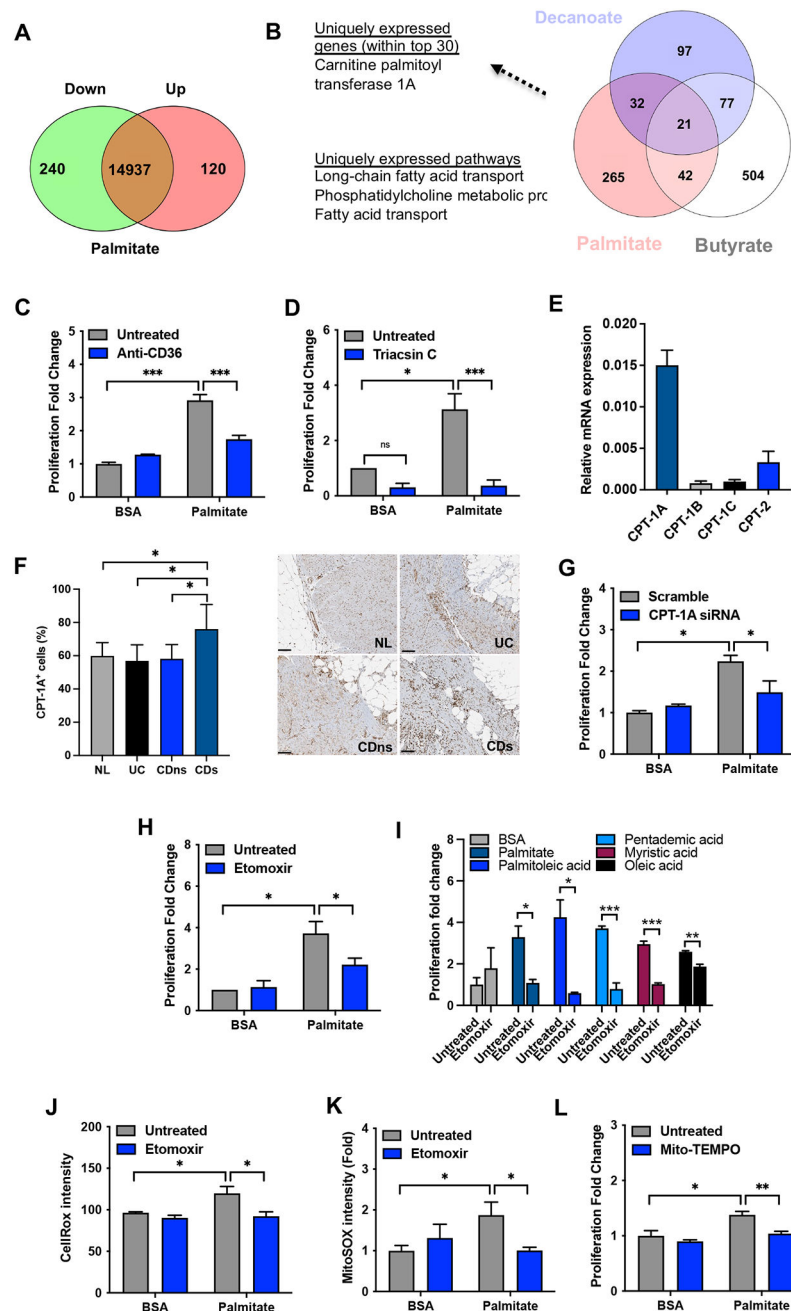


Figure 4. Palmitate induces primary human intestinal muscularis propria smooth muscle cell proliferation via fatty acid metabolism and uptake into mitochondria.

A. Next generation sequencing was performed in HIMC treated with 50 μ M palmitate, or control medium for 12 hours (n=8 per group). Venn diagram for up and downregulated genes. **B.** Next generation sequencing was performed in HIMC treated with 50 μ M palmitate, decanoate, butyrate or control medium for 12 hours (n=8 per group). Uniquely changed genes for each fatty acid underwent GO-pathway analysis and examples of Top 10 pathways and genes unique to palmitate are shown. **C.** HIMC proliferation in response to 50 μ M palmitate with or without addition of CD36 inhibitor Sulfosuccinimidyl oleate

(SSO) (50 μ M) for 48h. Proliferation was measured using 3H-thymidine. N=3. **D.** HIMC proliferation in response to 50 μ M palmitate with or without addition of the long chain acetyl-CoA synthetase (ACSL) inhibitor Triascin C (10 μ M) for 48h. Proliferation was measured using 3H-thymidine. N=3. **E.** CPT-1A, CPT-1B, CPT-1C and CPT-2 gene expression in the MP of human intestinal tissues relative to GAPDH. N=7 per group. **F.** Immunohistochemistry of CPT-1A expression in the muscularis propria (MP) of human intestinal resection tissues of normal (NL), ulcerative colitis (UC), non-strictured Crohn's disease (CDns) and strictured Crohn's disease (CDs). Quantification of CPT-1A positive cells relative to all cells using QuPath are shown. Scale bar: 100 μ m. N=6–7. **G.** HIMC proliferation in response to 50 μ M palmitate with or without selective siRNA knockdown of CPT-1A for 48h. Proliferation was measured using 3H-thymidine. N=3. **H.** HIMC proliferation in response to 50 μ M palmitate with or without addition of the carnitine palmitoyl transferase (CPT)-1 inhibitor etomoxir (50 μ M) for 48h. Proliferation was measured using 3H-thymidine. N=4. **I.** HIMC proliferation in response to 50 μ M of different long chain free fatty acids (FFA) with or without addition of etomoxir (50 μ M) for 48h. Proliferation was measured using EdU assay. N=3. **J.** HIMC reactive oxygen species (ROS) production in response to 50 μ M palmitate with or without addition of the carnitine palmitoyl transferase (CPT)-1 inhibitor etomoxir (50 μ M) for 24h. ROS was measured using CellROX Green Reagent. N=4. **K.** Mitochondrial reactive oxygen species (ROS) production in response to 50 μ M palmitate with or without addition of the carnitine palmitoyl transferase (CPT)-1 inhibitor etomoxir (50 μ M) for 24h were determined in HIMC. ROS was measured using MitoSox. N=4 technical replicates for one HIMC line. **L.** HIMC proliferation in response to 50 μ M palmitate with or without addition of mitochondria-targeted antioxidant mito-TEMPO (10 μ M) for 48h. Proliferation was measured using EdU. N=5. Abbreviations: CDs: Crohn's disease stricture; CDns: non-strictured Crohn's disease; UC: Ulcerative colitis; NL: normal tissue; CPT: Carnitine palmitoyl transferase. Data are presented as mean \pm SEM; ***P<0.001; **P<0.01; *P<0.05.

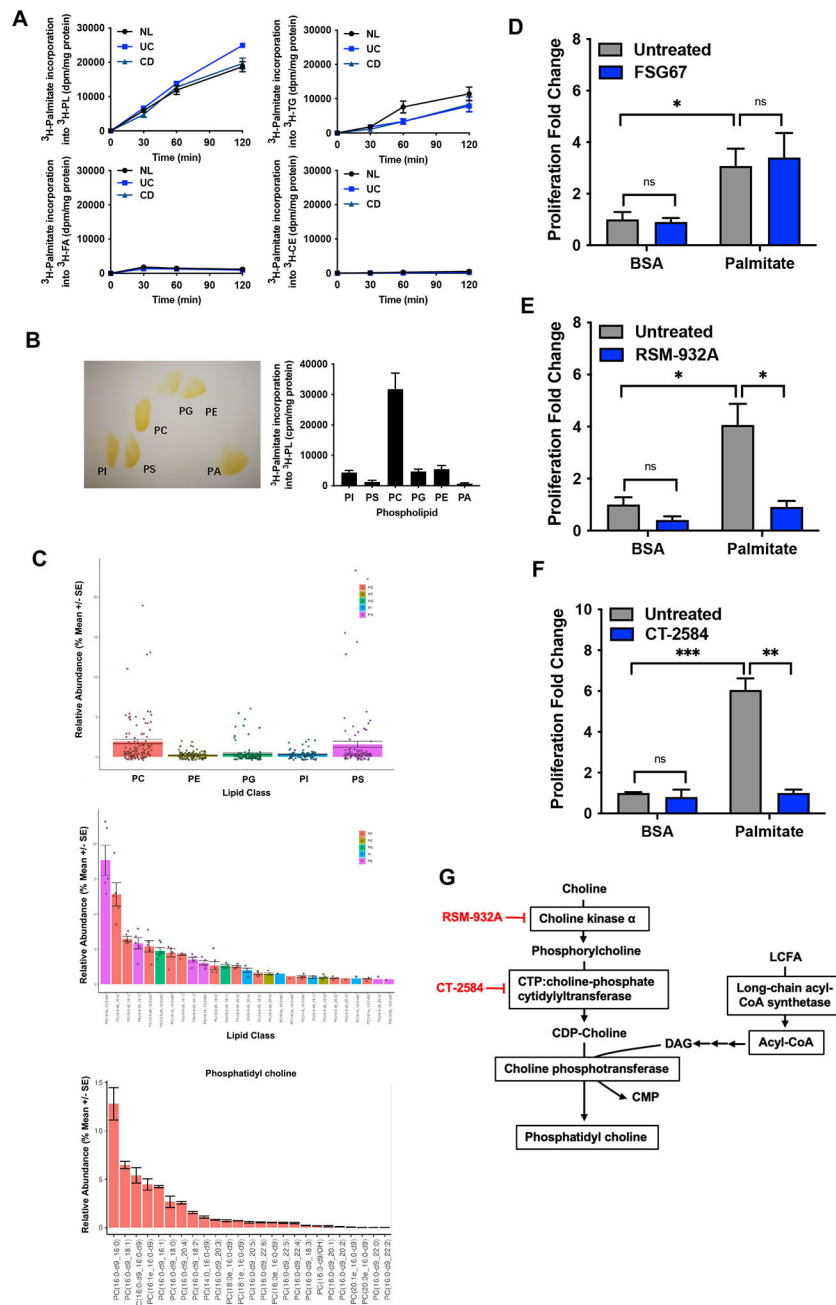


Figure 5. Palmitate induces proliferation of primary human intestinal muscularis propria smooth muscle cells via fatty acid metabolism.

A. Human intestinal muscularis propria smooth muscle cells (HIMC) derived from normal (NL), ulcerative colitis (UC) or Crohn's disease (CD) tissues were exposed to ^3H -labelled palmitate for 30 min, 60 min and 120 min and lipids contained in HIMC were extracted using chloroform/methanol. The extracted lipids were separated and the incorporation of ^3H -palmitate into phospholipids (PL), cholesteryl ester (CE), triglyceride (TG) and free fatty acid (FFA) was determined by the radioactivity quantified by liquid scintillation counting. N=4. **B.** Phospholipid analysis was performed using two-dimensional thin layer chromatography (2D TLC) with yellow spots corresponding to labeled lipids

including phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylserine (PS), and phosphatidylinositol (PI). Representative chromatography depicted on left and quantification using liquid scintillation counting depicted on right. N=3. **C.** HIMC were exposed to 50 μ M deuterium labelled palmitate for 1h, followed by lipidomic analysis. Top panel indicates relative abundance of the deuterated label by lipid class. Middle panel indicates relative abundance of the deuterated label ranked by individual lipids across lipid classes. Low panel indicates relative abundance of the deuterated label ranked by individual lipids within the phosphatidylcholine class. **D.** HIMC proliferation in response to 50 μ M palmitate with or without addition of the glycerol 3-phosphate acyltransferase (GPAT) inhibitor FSG67 (200 μ M) for 48h. Proliferation was measured using EdU assay. N=3. **E.** HIMC proliferation in response to 50 μ M palmitate with or without addition of the choline kinase α inhibitor RSM-932A (20 μ M) for 48h. Proliferation was measured using EdU assay. N=3. **F.** HIMC proliferation in response to 50 μ M palmitate with or without addition of the CTP:choline-phosphate cytidyltransferase (CT) inhibitor CT-2584 (2.5 μ M) for 48h. Proliferation was measured using EdU assay. N=3. **G.** Diagram depicting the discovered choline synthesis pathways in HIMC. Small molecule inhibitors are depicted in red font.

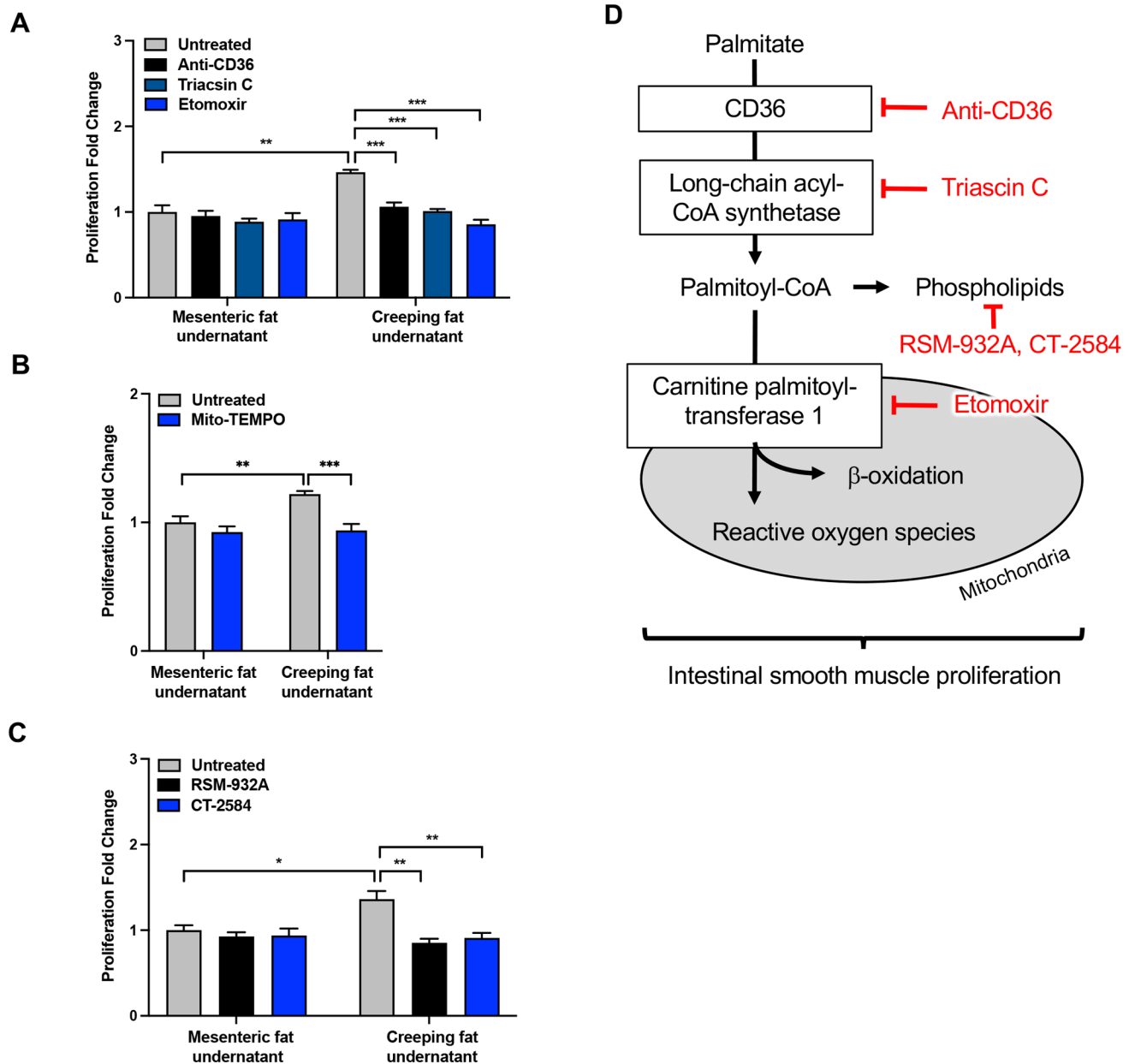


Figure 6. Creeping fat and non-creeping mesenteric fat conditioned medium induces proliferation of primary human intestinal smooth muscle cells via carnitine palmitoyl transferase 1 and fatty acid metabolism.

A. Cell culture medium was conditioned for 48h using 50 mg wet weight of creeping fat (CF) or non-creeping mesenteric fat (MF) from Crohn's disease. Subsequently conditioned medium was co-cultured with HIMC with or without the presence of the CD36 inhibitor Sulfosuccinimidyl oleate (SSO) (50 μ M), the carnitine palmitoyl transferase (CPT)-1 inhibitor etomoxir (50 μ M) or the long chain acetyl-CoA synthetase (ACSL) inhibitor Triascin C (10 μ M) for 48h and proliferation measured using EdU for 48h. N=4 per group.

B. HIMC were co-cultured with cell culture medium conditioned from creeping fat (CF) or non-creeping mesenteric fat (MF) of Crohn's disease with or without the presence of mitochondria-targeted antioxidant mito-TEMPO (10 μ M) for 48h and proliferation measured

using EdU assay. N=4 per group. **C.** HIMC were co-cultured with cell culture medium conditioned from creeping fat (CF) or non-creeping mesenteric fat (MF) of Crohn's disease with or without the presence of the choline kinase α inhibitor RSM-932A (20 μ M) or the CTP:choline-phosphate cytidyltransferase (CT) inhibitor CT-2584 (2.5 μ M) for 48h and proliferation measured using EdU assay. N=4 per group. **D.** Diagram depicting the discovered signaling pathways in HIMC. Small molecule inhibitors are depicted in red font.

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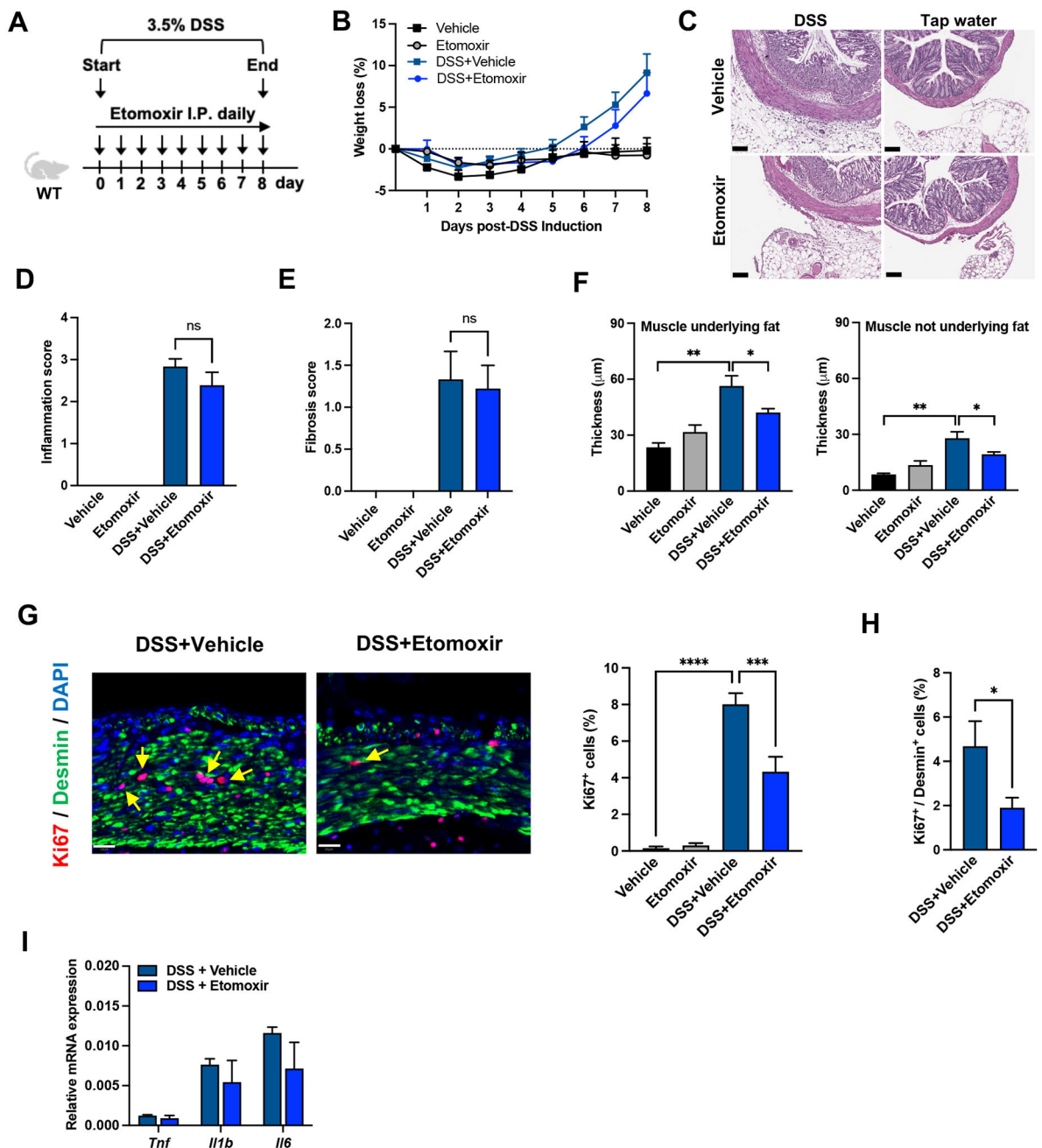


Figure 7. Administration of the carnitine palmitoyl transferase inhibitor etomoxir reduces muscularis propria thickness, but not inflammation or fibrosis in DSS colitis.

A. Experimental diagram for the DSS colitis model. Balb/c wildtype (WT) mice were subjected to administration of etomoxir or vehicle intraperitoneally (I.P.) daily starting the day of 3.5% dextran sodium sulfate (DSS) administration and all throughout the experiment.

B. The severity of DSS induced colitis was evaluated by measuring the body weight loss (N=5–9 per group). **C.** Colonic sections were fixed and stained with hematoxylin and eosin (H&E) for histological examination. Representative images are shown. Scale bar: 100µm. **D.** Colonic inflammation score was determined using H&E sections scored

by an IBD pathologist blinded to the experimental groups (N=5–9 per group). **E.** The severity of fibrosis was evaluated by an IBD pathologist blinded to the experimental groups using Masson trichrome staining (N=5–9 per group). **F.** Thickness of the colonic muscularis propria (MP) was measured separately for MP underlying mesenteric fat and the MP not underlying mesenteric fat (N=5–9 per group). Measurements were performed by QuPath software. **G.** Immunofluorescence for determination of percent cells positive for proliferation marker Ki67 in the colonic MP underlying mesenteric fat with a selective antibody to Ki67 (red), desmin (green) and DAPI (blue). Scale bar: 20µm. Representative Ki67⁺ cells are marked with yellow arrows. Quantification in bar graph. N=6 per group. Scalebar: 20 µm. **H.** Percent positive muscle cells (desmin) that also express the proliferation marker Ki67 indicating the majority of Ki67⁺ cells being muscle cells. **I.** Gene expression of inflammation genes in intestinal tissues relative to 18S ribosomal RNA. N=5 per group. Abbreviations: WT: Wildtype; DSS: Dextran sodium sulfate. Data are presented as mean ± SEM; ***P<0.001; **P<0.01; *P<0.05.