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DELINEATING THE ROLE OF FATTY ACID METABOLISM TO IMPROVE THERAPEUTIC STRATEGIES FOR COLORECTAL CANCER

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DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

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ABSTRACT OF DISSERTATION

DELINEATING THE ROLE OF FATTY ACID METABOLISM TO IMPROVE THERAPEUTIC STRATEGIES FOR COLORECTAL CANCER

Colorectal cancer (CRC) remains a leading cause of cancer-related deaths in the world, comprising over 1 million new cases each year and over 500,000 deaths. CRC, when detected at an early stage of disease development, can be effectively treated, with a 5-year survival rate of over 90%. Such standard treatments include surgical resection of the primary tumor in combination with adjuvant chemotherapy. However, even with advancements in surgical procedures and chemotherapeutic targets, when CRC progresses to a more advance stage, the 5-year survival rate decreases significantly to just under 14%. This stark decrease in patient survival rate can be directly contributed to the lack of effective chemotherapeutics as well as relapse, which occurs in 30-50% of patients treated for CRC and results in a far more aggressive disease. With such a large rate of CRC relapse and drastically low survival rates, the need for new therapeutic targets for the disease are particularly needed.

Dysregulation of fatty acid (FA) metabolism has been identified as a hallmark of cancer and a key proponent to CRC development and progression. FAs are used for a multitude of purposes within the cell, such as signaling molecules, components of membrane synthesis, and most importantly, as a direct energy source. Two different pathways contribute to FA utilization in cancer cells. First, FAs can be synthesized *de novo*. FA Synthase (FASN), a key enzyme of de novo FA synthesis, catalyzes the synthesis of palmitate from acetyl-CoA and malonyl-CoA. Another prominent pathway utilized by cells to obtain FAs is exogenous FA uptake via transmembrane FA transporters including FA Translocase (CD36). CD36 is a multifunctional glycoprotein, is primarily involved in the binding and transportation of low-density lipoproteins and long-chain/ultra-long-chain FAs. My research assessed the role of CD36 in CRC growth and metastasis as well as the relationship between CD36 and FASN, particularly, the aspect of this relationship when *de novo* lipid synthesis is inhibited using novel FASN inhibitors.

FASN upregulation has been previously shown to contribute to primary CRC growth and progression to metastasis. Chemical inhibition of FASN via a novel FASN inhibitor TVB-3664, has shown significant promise in the treatment of CRC *in vitro* by reducing CRC proliferation via a decrease is cellular respiration. Interestingly, pre-clinical evaluation of this inhibitor in patient derived xenografts (PDXs) *in vivo* suggests that high expression of FASN does not determine a positive response to FASN-targeted therapy and only approximately 30% of PDXs exhibit significant tumor reduction. These results suggest that there may be alternative pathways which may contribute to FASN inhibition resistance.

CD36 has been studied in various diseases, including glioblastoma, breast, ovarian, and oral carcinomas, where it has been found to significantly contribute to disease progression and metastasis. However, CD36 has not yet been thoroughly investigated in CRC, and has not been studied in relation to *de novo* lipid synthesis. My studies show that CD36 is upregulated in CRC primary and metastatic tumors and an increased expression level of CD36 is associated with an increase in FA uptake. Furthermore, CD36 inhibition significantly decreases proliferation of CRC established and primary cell lines *in vitro*, and knockdown of CD36 reduces subcutaneous xenograft tumor growth *in vivo*. Most excitingly, inhibition of FASN significantly and specifically upregulates CD36 expression, but not other FA transporters, in human tissues, established and primary cell lines, and genetically modified mice with heterozygous and homozygous deletion of FASN. This upregulation of CD36 in the presence of FASN inhibition resulted in a CD36 specific increase in the uptake of exogenous FA analogues, further supporting the role of CD36 as a potential compensatory mechanism to FASN-targeting therapy. Additionally, inhibition of FASN and CD36 in combination resulted in an additive effect on the reduction of CRC cell proliferation.

Late-stage metastatic CRC has a substantially lower rate of 5-year survival compared to earlier stage disease. As aforementioned, in addition to primary CRC cell survival and proliferation, CD36 was shown to be further upregulated in CRC metastatic tissues. My studies demonstrate that CD36 promotes CRC cell invasion and colony formation *in vitro.* Additionally, an increase in CD36 is associated with more metastatic CRC cell lines and promotes metastasis of CRC cells *in vivo*. Interestingly, CD36 expression is associated with matrix-metallopeptidase-28 (MMP28), the newest member of the matrix-metallopeptidase family of proteins. MMP28 is involved in the degradation of the extracellular matrix (ECM), particularly the disassembly of collagen fibers within cell-cell adhesions. MMP28 has been suggested in the metastasis of various diseases including lung and gastric cancer. MMP28 had not yet been investigated in CRC. Knockdown of CD36 decreases MMP28 expression and increases the expression of e-cadherin, a critical protein involved in cell-cell adherent junctions and whose loss is a well-known marker for epithelial to mesenchymal transition (EMT) in various cancers. Furthermore, knockdown of MMP28 is sufficient to significantly upregulate e-cadherin

expression suggesting that CD36 potentially regulates metastasis through early EMT via the upregulation of MMP28 and loss of e-cadherin.

New therapeutic strategies in the treatment of CRC are urgently needed to improve the survival rates of those patients with late-stage disease. Altered FA metabolism is a common characteristic of many cancers including CRC. The studies described here investigate the role of CD36 in CRC and upregulation of CD36 as a potential compensation mechanism to FASN-targeted therapy which is currently being tested in several clinical trials. The results of my studies demonstrate that CD36 may present a viable target for treatment of both primary and metastatic CRC. Furthermore, this study provides the rationale for targeting CD36 in combination with FASN inhibitors and demonstrates that inhibition of CD36 increases efficacy of FASN-targeted therapy via a reduction of the free-FA pools available to CRC tumor cells. Further investigation into the mechanistic functions of both CD36 and FASN in CRC as well as the contribution of CD36 to CRC metastasis is needed to provide more efficacious treatment strategies of CRC.

KEYWORDS: CD36, FASN, COLORECTAL CANCER, FATTY ACIDS, METABOLISM

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08/13/2021

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1. INTRODUCTION TO COLORECTAL CANCER, FATTY ACID METABOLISM, FATTY ACID SYNTHASE, AND FATTY ACID TRANSLOCASE

1.1 Colorectal Cancer

Colorectal cancer (CRC) is the leading cause of non-smoking related cancer deaths in the world, and the second leading cause of cancer related deaths, with estimates of more than 145,000 new cases in the United States, including more than 50,000 new deaths in 2020 [1, 2]. In 2018, diagnosed cases of CRC were over 1.8 million worldwide, with over 800,000 deaths, a number which accounted for almost 10% of all cancer related deaths in the world [3]. Current models predict that CRC diagnoses will increase to over 2.5 million annually by 2035 [4]. As with many cancers, CRC has historically been viewed from the perspective of a disease directly related to age. However, when looking at epidemiological studies over the past 40 years, incidence rates for CRC in younger age groups, those 20-49 years, has increased by over 47 % [5]. Patients within high-risk demographics are encouraged to receive asymptomatic screening for CRC as this has been shown to reduce mortality rates of the disease significantly through both the removal of lesions prior to disease development as well as earlier stage CRC diagnosis [6-9].

CRC, as with many of the oncogenic diseases, is heterogenous and can be classified into several molecular sub-types [10-12]. The CRC Subtyping Consortium, using six independent molecular classification systems, have recognized four unique molecular subtypes of CRC which are: Microsatellite Instability Immune (MII), Canonical, Metabolic, and Mesenchymal [10, 11, 13]. Of these subtypes, the most prevalent at 37% is the Canonical, which is characterized with marked wingless-related integration site (WNT) and MYC signaling activation. CRCs typical of the Canonical subtype exhibit an initial loss of the adenomatous polyposis coli (APC) gene followed by an activating mutation of the KRAS signaling pathway in combination with the loss of tumor protein-53 (TP53) [13, 14]. Clinically, nearly 40% of Canonical subtype CRC tumors are diagnosed at Stage III, however this subtype represents the highest overall 5-year survival rate at nearly 80% [10]. This is followed by the Mesenchymal subtype at 23% of all CRCs, exhibiting significant transforming growth factor beta (TGFβ) activation with stromal invasion, angiogenesis, and can be both with and without prominent Wnt signaling. Tumors of this subtype display pathologically serrated legions, with cells representing a mesenchymal phenotype [15]. Cancer associated fibroblast (CAFs) with increased TGF-β signaling profiles, as well as a prominent inflammatory microenvironment, are also common of this sub-type [10, 13]. These are followed up by the MII subtype at 14% and consisting of significant microsatellite instability and a pertinent immune system activation. The MII subtype is specifically represented pathologically by the presence of serrated polyp precursor legions, consisting of high BRAF mutation rates, loss of tumor suppressor function and missed-match repair (MMR) at the epigenetic level [16]. The combination of BRAF mutations and

MMR deficiency results in a hyper mutated genetic profile, with nearly a 20-fold increase in mutation rate per 10^6 base pairs compared to microsatellite stable tumors [10, 13, 16]. Lastly, the Metabolic subtype comprises 13% of all CRCs and possesses increased metabolic dysregulation, particularly in the DNA repair, glutaminolysis, lipogenesis and cell cycle pathways [10, 11, 13, 14]. More in-depth investigation into the various subtypes of CRC and their unique molecular profiles may increase treatment efficacy for CRC.

1.2 Treatment Strategies for CRC

As previously mentioned, CRC rates, although declining in those ages over 50, are increasing significantly in younger populations and CRC remains the second most common cause of cancer related deaths in the world [5, 17]. Those patients diagnosed with early stage CRC have nearly a 10 fold increase in 5 year survival rate when compared to later stage disease with more distant metastases [18, 19]. For those with earlier stage disease, the therapeutic approach is much simpler, relatively speaking, than those with later, more advanced CRC.

A typical treatment strategy for those with stage 0-I CRC is surgical excision of the cancerous lesion, typically contained within a polyp. In lesions not contained within a polyp, or if the edge of the polyp still contains cancerous cells, a partial colectomy may be recommended to remove the section of the colon with disease [20, 21]. Those patients with more progressed disease, including some stage II and stage III-IV CRC, surgical excision of the primary tumor and/or partial colectomy is recommended in combination with adjuvant chemotherapy. Such chemotherapeutic agents used in combination with surgical resection are non-targeted drugs and include 5-fluorouracil, leucovorin, oxaliplatin and capecitabine [20, 22]. 5-fluorouracil (5FU) was one of the earliest chemotherapeutic anticancer agents identified and is an antimetabolite, inhibiting the enzyme thymidylate synthase, thus preventing the production of thymidine and consequently impeding DNA synthesis and cell proliferation [23, 24]. 5FU is used across a wide range of diseases including breast, skin, and head and neck cancers [25]. Although it has seen some success in treating CRC, 5FU only produces significant response in <20% of patients when used alone, and for that reason it is typically used in combination with other therapeutics. [26, 27]. Combination of 5FU with leucovorin as well as oxaliplatin have seen increases in treatment efficacy [28]. Leucovorin, also known as folinic acid, significantly enhances the binding potential of 5FU with thymidylate synthase, increasing patient response to 26-43%, depending on treatment course [29]. Oxaliplatin is a third-generation platinum compound, with a unique 1,2-diaminocyclohexane moiety [30]. Analogous with other platinum-based therapeutics, oxaliplatin derived cytotoxicity is due to platinum induced DNA crosslinks, typically between two guanines, or a guanine and adenine base pair, resulting in inter- and intra-strand platinum-DNA adducts. Due to the 1,2-diaminocyclohexane moiety however, these adducts are significantly bulky, resulting in a more effective blocking of DNA-mismatch repair enzymes, and consequently, an increased level of cytotoxicity when compared to other traditional

platinum-based therapies [30, 31]. Oxaliplatin alone produces relatively low response rates in patients, approximately 20%, however, used in combination with the 5FU and leucovorin, patient response rates significantly increase, to nearly 50%, accompanied by an increase in progression free survival [32-34]. Together, this combined treatment regimen of oxaliplatin, 5FU and leucovorin is known as the FOLFOX regimen. Other regimens consisting of multiple chemotherapeutics agents in combination include FOXFIR, a mixture of 5FU and irinotecan, as well as XELOX, an oxaliplatin and capecitabine mixture. The efficacy of these varying regimens is comparable to each other [35]. Together these regimens remain the standard approach for first-line treatment of CRC.

Due to the high mortality of advanced stage disease, there are several ongoing efforts to establish effective targeted therapies for the treatment of CRC. There has been emphasis on targeting the epithelial growth factor receptor (EGFR) pathway including the protein EGFR, human epidermal growth factor receptors (HERs), v-raf murine sarcoma viral oncogene homolog B1 (BRAF), and encoding phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K). Within CRC patient populations, those patients with aberrant EGFR upregulation comprise up to 77% of the total CRC patients [36]. Furthermore, nearly 5% of CRC patients also exhibit HER2 overexpression [37, 38]. Of the targeted therapies for EGFR, those most prominent include the monoclonal antibody cetuximab and its fully humanized derivative panitumumab [39, 40]. These therapeutics work to induce EGFR degradation after biding to the receptor in its extracellular domain and have shown great promise as first-line treatment for CRC [35, 39]. However, 10% of CRC patients also exhibit oncogenic mutations in BRAF, which has been hypothesized to contribute to anti-EGFR therapeutic resistance [41]. Current treatment of CRC with mutant BRAF consists of using BRAF inhibitors, such as vemurafenib, in combination with upstream, EGFR inhibitors such as cetuximab [42]. This combination is necessary, as patients treated with BRAF inhibitors alone often see no significant change in progression-free survival [35, 43, 44]. Additionally, it is suggested that inhibition of EGFR can also upregulate the expression of HER2, which, due do similar downstream RAS and PI3K signaling, can compensate for EGFR inhibition [45]. To target HER2, a combinational treatment of trastuzumab and pertuzumab, or trastuzumab and lapatinib are utilized [46, 47]. PI3K mutations coincidentally can lead to downstream activation of AKT and resistance to EGFR inhibition. Currently, several studies are investigating potential inhibitors to PI3K to use in combination with EGFR targeted therapy, however much more understanding is needed for effective inhibition of PI3K within the clinical setting [35].

Another prominent pathway which has become the focus of targeted intervention with metastatic CRC is the vascular endothelial growth factor (VEGF) pathway, which is responsible for the regulation of angiogenesis [48]. Interactions between VEGF-A and vascular endothelial growth fact receptor 2 (VEGFR-2) result in the activation of downstream pathways including the previously mentioned RAS/RAF/ERK/MAPK and PI3K/AKT pathways. Those patients with VEGF-A

gene amplification, while composing a relatively small number of total CRC patients, have disease that is typically more aggressive and results in significantly lower 5-year survival rates [49]. The monoclonal antibody bevacizumab is currently the prominent antiangiogenic therapy targeting VEGF and still the only Food and Drug Administration (FDA) approved first-line therapy which targets VEGF. However, various new therapeutic agents are being developed and tested in phase II and III clinical trials with some being approved for second-line treatment of CRC [35, 50]. Due to the high complexity and varying pathway intersections involved in the VEGF pathway, resistance to VEGF targeted therapy can frequently be observed [35]. Strategies targeting VEGF in combination with other potential pathways of compensation, such as the fibroblast growth factor (FGF) and c-Met pathways as well as angiopoietin-2 signaling, have shown promise in pre-clinical studies [51, 52]. However, in some early clinical trials, several strategies have failed to yield significant results and more investigation is needed to improve treatment modalities involving the targeting of VEGF [53].

More recently, advances in understanding of the immune checkpoint pathway has led to development of multiple therapeutics which aim to enhance the recognition and clearance of cancer cells by the immune system [54]. Two prominent agents used to target the immune-checkpoint pathway in metastatic CRC were pembrolizumab and nivolumab [35]. These treatments are both monoclonal antibodies which target programmed cell-death protein 1 (PD-1) on the tumor cell surface and which, through binding to programmed cell-death protein 1-ligand (PDL-1), activates T-cell dysfunction and tolerance, resulting in an escape by the tumor cell from the surveillance of the immune system [55-57]. Both therapeutics earned FDA-approval after they displayed significant patient response in those patients whose tumors exhibit high microsatellite stability (MSI-H) [58, 59]. However, those patients with intact mismatch repair (MMR) showed little to no response to these immune-checkpoint targeted therapies. Several other potential treatments are currently under investigation and other therapeutic targets within the immune-checkpoint pathway are also being explored with the hopes of broadening the range of patients who respond well to this type of therapeutic strategy.

1.3 CRC Treatment Efficacy

Screening for CRC, which typically consists of a colonoscopy and is the first line of defense in the prevention of CRC, has drastically increased within the United States in recent years [60]. Those patients between the ages of 50-75 years have significantly increased their up-to-date screening rates since 2005, going from 46.8% to 68.8% in 2018 [61, 62]. Consequently, the incidence and mortality rates for CRC in these older populations have seen some decline since the implementation and higher participation in early CRC screening [63]. Despite this increase in screening and early CRC detection, late-stage CRC remains to be ever problematic and with this, disease relapse and mortality rates continue to remain high. Currently, only 36% of patients diagnosed with CRC have disease that has

not yet spread to distant tissues while 20-25% percentage of patients are diagnosed with late-stage metastatic CRC. [2, 64]. Those whose CRCs are diagnosed at an early stage (Stage I-III) have a significantly higher 5-year survival rate when compared to those diagnosed with much more progressed disease (Stage IV), which has a 5-year survival rate of less than 14% [5, 8, 65] **(Table 1.1).** Due to this significant reduction in survival of late-stage CRC, emphasis on discovering new therapeutic strategies for late-stage CRC is necessary.

Achieving complete remission, or more specifically pathological complete response (pCR), which is the complete absence of cancerous tissues via biopsy or surgical resection, has been shown to significantly improve patient 5-year survival. Those who do achieve pCR have an average 5-year survival rate of 83% compared to those patients who do not, which have an average 5-year survival rate of only 66% [66]. Those who are diagnosed with early stage, localized CRC see almost 100% pCR after surgical resection [67]. Historically, those diagnosed with later stage, invasive and/or metastasized CRC would likely only receive minor, palliative primary surgical resection due to their increased tumor burden, which is typically present with significant liver metastasis [67]. Due to advances in new therapeutic approaches, patients with Stage II and III CRC can receive neoadjuvant therapy; that is therapy which is administered pre-operatively to reduce tumor burden to an operational size and can exhibit almost a 25% pCR rate [68]. Those diagnosed with Stage IV disease, even if qualifying for curative surgery, still exhibit a poor response rate [68]. This discrepancy between patient groups can be contributed to the lack of pCR and the heterogeneity of metastatic burden observed between different late stage CRC patients, resulting in a significantly higher rate of disease recurrence [69].

Although several strategies have been employed to detect CRC recurrence early, where it can be more effectively treated, disease recurrence remains high [70]. Of all CRC patients treated, approximately 40% experience some sort of recurrent disease [71]. More specifically, of all patients diagnosed with at least potentially curable disease, up to 60% will relapse, often with more difficult-to-treat metastatic disease [70]. The most critical period in which recurrence can occur is 3 years following completion of definitive care [72]. Strategies currently used to aid in the early detection of recurrent CRC which have important clinical significance include blood tests to monitor liver enzyme levels and regular imaging of both the liver, the most common site of metastasis in CRC, and the lungs [70, 73, 74]. For example, if monitored closely, up to 20% of recurrent liver metastasis can be successfully surgically removed, with significant increases in patient survival [75-77]. However, a large proportion of patients diagnosed with CRC could greatly benefit in better preventative and detection strategies against recurrent CRC.

CRC patients diagnosed with local advanced, or late-stage disease are still in need of more effective therapeutic strategies. More effective therapeutic targets could improve definitive treatment of both primary and metastatic CRC as well as assist in the prevention of disease recurrence. Recently, alternative pathways

including that of cellar metabolism, specifically altered fatty-acid metabolism, have been implicated in CRC progression and metastasis [78]. Dysregulation of lipid metabolism is a hallmark of cancer; however further understanding of the intricacies in this complex metabolic pathway is required to identify appropriate therapeutic targets for CRC.

1.4 Fatty Acids and Fatty Acid Metabolism

1.4.1 Fatty Acid Structure and Distribution

Fatty acids (FAs) consist of a carboxylic acid which contain a long, aliphatic side chain. This side chain of carbons can vary in number typically from 2-26 [79]. Categorically, FAs can be grouped in four groups based on the length of their aliphatic side chain: short chain (2-6 carbon atoms), medium chain (8-12 carbon atoms), long chain (14-18 carbon atoms), and very long chain (20-26 carbon atoms) [79, 80]. FAs can be defined as saturated (SFA) or unsaturated (UFA) where SFAs consist of molecules with no carbon-carbon double bonds, meaning the entire molecule is completely saturated with hydrogen atoms aside from the carboxyl group. UFAs contain at least one carbon-carbon double bond [81]. The most common types of FAs found within mammals are the long-chain and very-long-chain FAs, which include palmitic and oleic acid [80].

Typically, FAs are not found in most organisms as stand-alone molecules, but instead, they are most often in the forms of either cholesteryl esters, phospholipids, or triglycerides. Cholesteryl esters are a FA bound to a cholesterol molecule via an ester bond between the carboxyl group of the FA and the hydroxyl group of the cholesterol. These molecules, when hydrolyzed at the ester bond, release a free FA (FFA) and the cholesterol molecule for an energy source or membrane and lipoprotein formation [82]. Phospholipids contain a hydrophilic phosphate group bound to a hydrophobic region consisting of one or two FAs, which are all bound to each other via a glycerol molecule [83]. These molecules are then used for structural purposes, specifically membrane synthesis throughout the cell. Triglycerides (TGs) are esters formed by a glycerol molecule and three FAs. TGs are the primary component of fat within the body [81]. As with FAs alone, TGs can be considered saturated or unsaturated. The FA chains contained within the TG can all vary in length, but typically consist of either 16, 18, or 20 carbon atoms. Primarily, TGs are metabolized by a member of the pancreatic lipase family of enzymes, which hydrolyzes the ester bond within the TG, releasing the three FFAs and the glycerol molecule in a process known as lipolysis [81]. These FAs can then be readily absorbed in the intestine and utilized by the cells [84].

1.4.2 Fatty Acids as Building Blocks of Cellular Membranes

The most abundant and fundamental building block of all cellular membranes are phospholipids [85]. These critical components account for 50-60% of the total membrane lipid composition [85]. Phospholipids, because they contain

both hydrophilic and hydrophobic properties (also known as amphiphilic), can form lipid bilayers [86]. The FA component of these phospholipids form the hydrophobic tails, which due to entropy, cause the spontaneous formation of the bi-lipid layer in aqueous solutions [85]. Typically, the phospholipid consists of two FA tails, one of which is unsaturated with one or more double bonds, and the other is saturated, with no double bonds present [83, 87]. This heterogeneity allows the numerous FA tails of the phospholipids to arrange in such a way spatially that they are tightly packed yet still fluid relative to each other [87]. The fluidity of the bi-lipid layers is critical to the normal function of various proteins which are embedded within the membrane as well as the diffusion of other biomolecules [88]. Phospholipid molecules can diffuse laterally within the same monolayer, as well as flip-flop across monolayers, although the flip-flop is much less common [87]. To facilitate the movement of new phospholipid molecules across the two monolayers for the purpose of new membrane synthesis and membrane repair, the cell utilizes phospholipid translocator enzymes to catalyze the process [87]. The degree of fluidity of the membrane can also vary based on the variations in unsaturation of the FA tails. More unsaturated tails, containing more double bonds, allow for a more fluid membrane due to the spatial requirement of the double bonds and thus the less densely packed lipid molecules [87]. The unique dynamics of the phospholipid bilayer allow the cellular membranes to be incredibly diverse while serving as a strong barrier for the entire cell from its external environment, as well as separating the various compartmental organelles and the nucleus within the cell [85, 86].

Alongside phospholipids, FAs also contribute to the formation of numerous other types of lipids found within cellular membranes including sterols (primarily cholesterol), and sphingolipids [89]. Sterols, with cholesterol being the most prominent type in mammalian cells, comprise nearly 30% of the cellular membrane [90]. Cholesterol can be synthesized *de novo* or transported and internalized in the form of low-density lipoproteins (LDLs) [91].Cholesterol contributes significantly to the structural integrity of cell membranes as well as helps mediate membrane fluidity [92, 93]. Sphingolipids are lipids which are composed of a spingoid base backbone, a backbone comprised of aliphatic amino alcohols, the most common being sphingosine, attached to a FA via an amide bond [94]. Sphingolipids contribute structurally to cell membranes as well as serve as cell signaling molecules [95]. Sphingolipids can frequently associate with cholesterol within the cell membrane to form lipid rafts [96]. These relatively more-dense areas of the cell membrane can contain various signaling proteins and contribute to overall membrane fluidity [97, 98]. It is proposed that lipid rafts facilitate cell signaling by providing spatial arrangements of various proteins, allowing for more precise protein-protein interactions and thus contributing to numerous signaling cascades [99].

1.4.3 Fatty Acids as Signaling Molecules

Furthermore, aside from membrane formation, FAs can be utilized as lipid signaling molecules. Such molecules include prostaglandins, which are synthesized using arachidonic acid (AA), the 20-carbon unsaturated fatty-acid, and are key mediators of inflammatory responses to infection or injury [100, 101]. Other signaling molecules synthesized from AA, also known as eicosanoids, include thromboxane's and leukotrienes, which are responsible for blood clotting and immune function regulation, respectively [102].

As previously mentioned, sphingolipids, aside from facilitating cell signaling through lipid rafts, can themselves also serve as signaling molecules within the cell [95, 99]. Sphingolipids, particularly their metabolites ceramide, sphingosine, and sphingosine-1-phosphate, contribute to the signaling of cellular differentiation, cellular apoptosis and proliferation [95]. Ceramide, produced by the hydrolysis of sphingomyelin by sphingomyelinase, is typically the primary secondary messenger in sphingolipid dependent cell signaling [103]. Ceramide has been shown to be directly related to cellular apoptosis [104]. Conversely, sphingomyelin synthase facilitates the synthesis of sphingomyelin from ceramide, together forming the sphingomyelin cycle [105]. The phosphorylation of sphingosine by the two kinases sphingosine kinase 1 and 2 (SPHK1 and SPHK2) yields sphingosine-1-phosphate (S1P) [106]. S1P is involved in various cell signaling pathways including cell growth and suppression of apoptosis [107, 108]. Interestingly, S1P directly mediates apoptosis suppression through the inhibition of ceramide signaling, thus creating a well-regulated balance in which intracellular levels of ceramide and S1P contribute to overall cell fate [108].

In addition to the aforementioned FA derivatives involved in cell signaling, FFAs themselves can be utilized as cell signaling molecules [109]. FFAs can stimulate the transcription of numerous genes involved in various metabolic pathways. SFAs can stimulate genes within the sterol-regulatory element binding protein 1 (SREBP1) pathway, which in turn is responsible for the synthesis of other FAs and cholesterol [110, 111]. UFAs can however stimulate fatty-acid oxidation and in turn prevent further production of new UFAs [112].

1.4.4 Fatty Acids as An Energy Source

As previously mentioned, FAs are an important source of energy regarding cellular metabolism [79, 80, 102]. FAs have nearly twice the yield of energy compared to carbohydrates [113]. The key element of FAs which make them a prospective energy source for cells is their long aliphatic side chain. When a FFA is present within the cell, the primary method in which they are utilized for energy is FA beta-oxidation (FA β-oxidation), which describes the formation of acetyl-coenzyme-A (acetyl-CoA) molecules via the breakdown of long chain FAs [79, 114-116].

For a FA to first enter the FA β-oxidation pathway, a coenzyme-A (CoA) group is added to the FA via a thioester bond resulting in a fatty acyl-CoA molecule.

This reaction is also known as FA activation, and is catalyzed by a cytosolic acyl-CoA synthase(ACS) enzyme [117]. The specific acyl-CoA synthase used is dependent on the length of the FA. FA β-oxidation occurs within the mitochondria, however long chain FAs and fatty acyl-CoA molecules cannot freely translocate across the mitochondrial inner and outer membranes [79, 114, 116]. To overcome this limitation, a specific transport mechanism, the carnitine shuttle system, is utilized. A carnitine molecule is added to the newly formed fatty acyl-CoA molecule by carnitine palmitoyl transferase 1 (CPT1) on the surface of the outer mitochondrial membrane. The resulting fatty acylcarnitine can then be transported across the outer mitochondrial membrane and into the intramembranous space between the inner and out membranes [114, 116]. To then cross the inner mitochondrial membrane, the fatty acylcarnitine is transported via acylcarnitine translocases. Once across the inner mitochondrial membrane, carnitine palmitoyl transferase 1 (CPT2) removes the carnitine molecule from the fatty-acyl-CoA, and the carnitine is shuttled back across the inner and outer membranes to be recycled for further FA transport [114, 116]. Of these phases, the transfer of carnitine to the fatty-acyl-CoA by CPT1 is the rate limiting step, with malonyl-CoA acting as an inhibitor of CPT1, reducing FA β-oxidation [114]. In periods of fasting, malonyl-CoA levels fall, favoring FA transport across the mitochondrial membrane and increased beta oxidation [80, 114].

Once the fatty acyl CoA is completely inside the mitochondria, FA β-oxidation can occur. The first step in FA β-oxidation is the removal of two hydrogen molecules from the beta and gamma carbons of the acyl-CoA by acyl CoA dehydrogenase and an flavin adenine dinucleotide (FAD) molecule [114-117]. This results in the production of one $FADH_2$ molecule and a 2-trans-enoyl CoA. The 2-trans-enoyl CoA is then hydrated across the same two carbons by enoyl-CoA hydratase, resulting in an L-β-hydroxyacyl CoA. The resulting hydroxyl group within the L-β-hydroxyacyl CoA is oxidized by 3-hydroxyacyl-CoA dehydrogenase and a nicotinamide adenine dinucleotide (NAD^+) molecule resulting in β-ketoacyl CoA and an NADH [114-117]. Lastly, the β-ketoacyl CoA is cleaved by a thiol group of another, independent CoA molecule, by β-ketothiolase between the beta and gamma carbons. This results in a single acetyl-CoA molecule consisting of the alpha and beta carbons of the original fatty acyl-CoA, and a new fatty acyl-CoA molecule that is two carbons shorter [114-117]. This process then repeats itself until the final four carbons from the original acyl-CoA chain are cleaved into two acetyl-CoA molecules [114-117]. Therefore, for each cycle of FA β-oxidation, one acetyl-CoA molecule is produced along with one $FADH_2$ and one NADH. The acetyl-CoA can then enter the tricarboxylic acid cycle (TCA) where it is oxidized to form three more NADH molecules as well as one more $FADH_2$ and a single adenosine triphosphate (ATP), which is the primary molecule used for energy within the cell. The NADH and $FADH_2$ molecules are then further utilized within the electron transport chain, where each NADH molecule can generate 3 ATP and each FADH2 molecule can generate 2 ATP [118].

1.5 Fatty Acids Acquisition: Synthesis vs. Uptake of Dietary FAs

There are multiple pathways in which cells can acquire FAs **(Figure 1.1)**. When FAs are released from fat tissue, more specifically from triglycerides via lipolysis as described previously, the FFAs are then transported throughout the circulatory system, typically chaperoned by albumin [119]. In this form, FAs can then be incorporated into most cell types within the body via specific FA transporters. Such FA transport molecules include the FA transport family of proteins (FATPs), FA binding proteins (FABPs), and FA translocase (CD36) [120]. These FFAs are then typically utilized as an energy source via FA beta oxidation but can also be used for a variety of purposes as mentioned above.

Alternatively, cells may synthesize FAs *de novo* through the *de novo* FA synthesis pathway. This pathway describes the method in which acetyl-CoA and malonyl-CoA, are converted into long-chain FAs [121]. This process involves the critical enzymes acetyl-CoA carboxylase (ACC) which carboxylates acetyl-CoA to form malonyl-CoA, and FA synthase (FASN), which synthesizes long chain FAs from malonyl-CoA [122]. FAs synthesized via *de novo* lipid synthesis are then utilized for both membrane construction and as an energy source.

Both pathways in which FAs can be obtained or synthesized are the major contributors to FA metabolism and are well regulated. The sterol regulatory element-binding proteins (SREBPs) are important transcription factors in the regulation of genes involved in both *de novo* lipogenesis and the uptake of exogenous cholesterol [123, 124]. Such genes involved in the SREBP regulation of de novo lipid synthesis include citrate carrier protein solute carrier family 25 member 1 (SLC25A1), which specifically contributes to glucose-mediated lipid synthesis by shuttling citrate from inside the mitochondria across the mitochondrial membrane and into the cytosol where it serves as a precursor to *de novo* FA and cholesterol synthesis [125, 126]. In relation to the regulation of exogenous FA uptake and utilization, the peroxisome proliferator-activated receptor (PPAR) family of proteins have been shown to be involved [127]. In liver and intestinal tissues, increased activation of PPARα increases transcription of FATPs and ACSs, which are mediators of FA transport and activation [127]. Additionally, PPARγ activates the transcription of FATPs and ACS in adipose tissues, suggesting that increases in exogenous FA uptake in dependent on tissues specific signaling cascades[127]. The regulation of other important proteins involved in *de novo* lipid synthesis and exogenous FA uptake, such as FASN and CD36, is still being investigated and much work is left to be done.

1.6 Fatty Acid Metabolism Dysregulation and Cancer

Altered FA metabolism has long been established as a hallmark of cancer [128-130]. Cancer cells are typically rapidly dividing and require significantly larger amount of energy to sustain their growth. FAs, as both an energy source and a

primary component of lipid membranes, which are required for newly dividing cells, are a prime resource for cancer cells [123, 131]. Specifically, cancer cells have been shown to utilize FAs to sustain proliferation and meet their energy demands in times of high metabolic stress [122].

Increased levels of FAs and FA metabolism have been shown to promote cell proliferation and disease progression in multiple cancers [78, 123, 132]. For example, ATP citrate lyase (ACLY) is an important enzyme linked to glucose metabolism and is involved in lipid synthesis through the conversion of citrate to oxaloacetic acid and acetyl-CoA [133]. ACLY promotes the growth of lung carcinoma cells in vitro and is a poor prognostic factor for patients with non-small cell lung cancer [134, 135]. Furthermore, ACLY has also been implicated in the tumor progression and as a poor prognostic factor in other cancers including prostate, breast, bladder, and hepatocellular carcinoma [136-139]. Conversely, proteins involved in the uptake of exogenous FAs have also been implicated in the promotion of cancer [123]. The low-density lipoprotein receptor (LDLR) family of proteins are responsible for the binding of exogenous LDLs and internalizing them [91]. These receptors have been shown to be upregulated and significantly contribute to tumor growth in glioblastoma [140]. Additionally, various members of the FATPs and FABPs have been implicated in cancer progression, including FABP4 in the promotion of breast cancer tumor proliferation and FATP4 in the tumorigenesis and tumor progression of renal cell carcinoma [141, 142].

Other proteins have been shown to be involved the dysregulation of FA metabolism in numerous diseases via the regulation of *de novo* lipid synthesis and exogenous FA transport [123, 124, 132]. The SREBP family of proteins have been shown to promote tumorigenesis, cell proliferation, and disease progression in numerous cancers including glioblastoma, renal cell carcinoma, hepatocellular carcinoma, hepatocellular carcinoma, prostate cancer, and CRC [143-146]. For example, SREBP1 and SREBP2 were found to contribute to CRC glycolysis, FA oxidation, and mitochondrial respiration and inhibiting both transcription factors lead to a reduction in CRC cell proliferation [143]. Additionally, SREBP-1 upregulates LDLR signaling in glioblastoma [140]. However, there is some controversy over the regulation of some FA metabolism proteins and cancer progression, including that of PPAR regulation of FABPs. PPARα induced expression of FABPs has been shown to both correlate with hepatocellular carcinoma progression as well as have antitumorigenic effects [147, 148]. Further understanding of the intricacies of FA metabolism regulation is required if new therapeutic targets are to be properly identified.

In CRC, aberrant FA metabolism and increased levels of FAs have been shown to promote CRC cell proliferation and disease progression [78]. Understanding mechanisms of dysregulated lipid metabolism and identifying potential therapeutic targets within this pathway are potential strategies in developing new therapeutic approaches for CRC. Interestingly, no work has previously looked at the relationship of *de novo* lipid synthesis with exogenous FA

uptake in CRC. CD36 is suggested to be involved in the progression of glioblastoma, oral, ovarian, and prostate cancer while FASN has been implicated in the promotion of CRC progression and metastasis [149-154], but the crosstalk between these two proteins has not been described before. My work provides new insight on the interconnection of these proteins during CRC progression and metastasis and provides the rationale for developing new therapeutic strategies targeting lipid metabolism in CRC.

1.7 FASN Structure and Regulation

FASN not a single enzyme, but in fact is a homodimer of two identical polypeptide subunits, with each subunit containing an entire system of seven enzymatic domains [155]. The enzyme contains three catalytic domains within the N-terminus which are the ketoacyl synthase (KS) domain, the malonyl/acetyltransferase (MAT) domain, and the dehydrase (DH) domain [155, 156]. The enzyme also consists of four separate domains in the C-terminus: the enoyl reductase (ER), ketoacyl reductase (KR), acyl carrier protein (ACP) and the thioesterase (TE) domains [155, 156]. Those enzymes at the N-terminal domain are primarily responsible for the binding of substrates, which are acetyl-Coa and malonyl-CoA. Those enzymes contained within the C-terminal domain are responsible of reduction of the newly forming FA precursor, and ultimately release of palmitate by the TE domain [156]. The ACP domain, which contains a prosthetic 4'-phosphopantetheine group, is responsible for the shuttling of all intermediates throughout the FA synthesis process [157, 158]. There are currently multiple models which predict the exact molecular structure of FASN. The current model of FASN arrangement suggests that the two identical subunits are arranged antiparallel, thus resulting in two active centers that are composed of the KS domain, responsible for chain elongation, and the ACP, KR, and ER domains, which are responsible for reduction. Therefore it is understood that FASN has two active centers that can act independently of each other and operate simultaneously [159].

It has previously been shown that FASN is strictly regulated by nutritional and hormonal stimuli [160, 161]. It has been shown that high-carbohydrate diets and increased blood insulin levels significantly upregulate FASN expression [162]. Regulation of FASN is not known to be due to covalent modification or due to allosteric regulation. Instead, the regulation of FASN is primarily at the transcriptional level [163]. FASN contains a 147-base pair (bp) region within its 5' promoter in which a sterol regulatory element (SRE), a specific sequence of 5′-ATCACCCCAC-3′ in which SREBPs can bind, is present at the150bp [164-167]. Along with this SRE, there are two regions containing an enhancer-box (E-box), one at the 65bp and another more upstream at the 332bp [165]. E-boxes are specific DNA sequences of CANNG which are recognized as binding sites for various transcription factors. The transcription factors specifically responsible for transcriptional regulation are upstream stimulatory factors (USFs) and SREBP-1a [163-166]. USFs are members of the helix-loop-helix leucine zipper family of

transcription factors [161]. USF1-2 heterodimers binding to the 65bp E-box is necessary for insulin mediated FASN transcription however further binding of USF1-2 to the 332bp E-box confers full activation of the gene [166]. Additionally, SREBP-1a, through interactions with the SRE at the 150bp and in combination with USF binding the two E-boxes, can further activate FASN transcription [165].

1.8 FASN Function

FASN is a key enzyme of *de novo* FA synthesis. FASN is crucial for the synthesis of palmitate, the 16 carbon long-chain saturated FA from both acetyl-CoA and malonyl-CoA [155]. The synthesis of FAs via FASN is an exceptionally complicated process, which involves each of the seven enzymatic domains of the protein [155]. As mentioned previously, each monomer of the homodimer which comprises FASN can function independently and simultaneously to synthesize FAs [159]. Within a single monomer, the synthesis begins with the transfer of acetate from acetyl-CoA to the KS domain along with the transfer of malonate from malonyl-CoA to ACP by the MAT domain [155, 168]. The KS domain then catalyzes the condensation of acetate and malonate to form acetoacetyl-ACP and CO2 [155, 169]. Next, the acetoacetyl group is reduced by the KR domain to form B-hydroxyacyl-ACP which requires an NADPH molecule. The DH domain then catalyzes the dehydration of β-hydroxyacyl-ACP followed by another reduction by the ER domain, forming a saturated four carbon FA attached to ACP [155, 157, 168, 169]. The KS domain then transfer the saturated fatty acyl from the ACP to itself, resulting in a net gain of two carbons to the acyl chain for each cycle of elongation. The now free ACP can renter the cycle, and after a total of six cycles, a single ACP attached to a palmitate (C:16) is synthesized. The final domain, the TE domain, the hydrolyzed the synthesized palmitate from the ACP, resulting in a free palmitic acid [155, 157, 158, 168, 169].

Typically, FASN is found within the normal tissues of the liver and adipose, where more lipogenesis occurs and is expressed at relatively lower levels in most other normal tissues [159]. FASN can mediate energy homeostasis by synthesizing lipids for the purpose of storage during periods of excess food intake [170]. Furthermore, FASN can play an important role in membrane synthesis and homeostasis and thus cell division via the utilization of palmitate to form very-long-chain FAs, which can then be utilized for the formation of glycolipids and sphingolipids as well as ceramides [170, 171].

Overall, FASN functions as an important mediator of FA metabolism within the cell. By being a sensor to nutrient availability as well as an intermediate contributor of membrane synthesis, FASN is significant within several crucial pathways within a cell. Most importantly, FASN is an incredibly complex protein, consisting of several unique enzymatic domains responsible for the synthesis of FAs, making it a critical component of *de novo* FA synthesis. However, more research is needed to further understand the diverse set of downstream signaling pathways in relation to FASN-mediated *de novo* FA synthesis.

1.9 FASN in CRC

Due to FASN's critical role in *de novo* lipid synthesis in various tissues and since altered FA metabolism is a well-established hallmark of cancer and is observed in CRC, it was possible that FASN may have a role in the development and progression of CRC [128-130]. Indeed, it has been shown that FASN expression is elevated in CRC primary tumors and FASN expression is further elevated in later stage disease and is a poor prognostic factor for patient survival [153, 172]. More specifically, it has been shown that inhibition of FASN reduces signaling associated with CD44, a cell surface protein involved in several pathways including cell proliferation, migration and angiogenesis and has been implicated in tumor progression and metastasis of multiple cancers, including CRC [153, 173]. Additionally, FASN was found to directly play a role in the maintenance of energy homeostasis in CRC by increasing *de novo* lipid oxidation [154]. Also, FASN has been implicated in the inhibition of autophagy in CRC through the accumulation of p62, a substrate which is degraded during autophagy, although more investigation is needed to delineate a specific mechanism [154]. Furthermore, FASN has been shown to play an important role in CRC cell proliferation and survival *in vitro* via the regulation of the AMP-activated protein kinase (AMPK)/ mammalian target of rapamycin (mTOR) pathway [172]. Together, the current literature supports the suggestion that FASN enhances CRC progression through increased FA metabolism, cellular respiration and alteration of oncogenic signaling.

1.9.1 FASN as a Chemotherapeutic Target in CRC

The striking upregulation of FASN in CRC and its role in CRC progression and poor prognosis make it a desirable target for therapeutic intervention. Multiple chemical inhibitors of FASN have been developed, including C75 and cerulenin [174-177]. C75 interacts with the KS domain of FASN and is a weak irreversible inhibitor [176]. Cerulenin is an antibiotic which was one of the earliest discovered inhibitors of FASN and interacts with the KS domain [177, 178]. Although C75 and cerulenin both showed promising anti-tumorigenic effects and increased cell death *in vitro*, both drugs induced significant side effects including weight loss and reduced nutrient consumption in mice [179]. These complications prevented these drugs from becoming more prominent therapeutic targets for CRC patients.

Another drug, which is a potent FASN inhibitor, is orlistat [180]. Orlistat is FDA-approved for the treatment of obesity and has shown antiproliferative properties against prostate cancer cell lines *in vitro* [180]. However, antitumorigenic effects of orlistat were less significant in xenograft models, and the drug is poorly soluble and does not easily cross the cell membrane, making it a less feasible therapeutic for CRC [181]. The repeated failures of multiple therapeutics emphasize the need for novel therapeutics with the potential to successfully and safely target FASN. One novel chemical inhibitor which has been developed to target FASN and has shown promise is TVB.

TVB compound have been developed by 3V-Biosciences, now known as Sagimet Biosciences, and is a potent reversible inhibitor of FASN [182]. TVB has a lower molecular weight and increased solubility compared to that of other FASN inhibitors mentioned above [183]. TVB acts through the reversible binding to the KR domain of FASN, inhibiting the first reduction of the acetoacetyl-ACP during FA synthesis [184].There are several analogs of TVB: TVB-2640 is used for clinical trials and their analogs TVB-3166 and TVB-3664 are used for pre-clinical studies *in vitro* and *in vivo* [185]. TVB-3166 has shown anticancer effects on oral squamous cell carcinoma cell lines as well as cell lines derived from lung, ovarian, breast, pancreatic, prostate, and hematopoietic cancers [184, 186]. In relation to CRC, TVB-3664 has demonstrated impressive anti-tumor activity and significant reduction of CRC established and primary cell line proliferation *in vitro* [185]. Increased expression of FASN was indicative of increased sensitivity to TVB-3664 *in vitro* [185]. Furthermore, the effect of TVB-3664 on CRC tumor growth *in vivo* was studied using patient derived xenografts (PDXs). PDX models are useful tools for preclinical drug evaluations because PDX tumors retain much of their parental host properties when implanted into mice, including their microenvironment and heterogeneity [187-190]. When various CRC PDX models, which tested positive for FASN expression, were analyzed for TVB sensitivity *in vivo*, 30% of PDXs responded positively, with up to 50% reduction in tumor weight [185]. However, 40% of PDXs treated had little to no response to TVB-3664 treatment, and two of the PDX models exhibited a negative response to TVB-3664 [185]. This then suggests that FASN expression alone is not sufficient to predict sensitivity to TVB treatment *in vivo*.

To further emphasize the clinical significance of targeting *de novo* FA synthesis using TVB, currently there are multiple Phase I/II clinical trials open using TVB analogs [191-193]. These include studies in lung and breast cancer as well as CRC [191-193]. Currently, TVB is in a Phase I clinical trial for CRC and it is yet to be seen how patient tumors respond to treatment [192]. However, with pre-clinical indications that not all patients will respond positively to TVB treatment alone, an investigation into potential alternative pathways which may contribute to TVB resistance is needed [185]. Due to FASN's critical role in *de novo* FA synthesis and FA metabolism, other proteins involved in FA metabolism maybe be suitable targets, such as those involved in the uptake of exogenous FAs, including CD36.

1.10 CD36 Structure and Regulation

CD36 is a 53kd transmembrane protein, which contains two intracellular domains, one at both the C and N-terminus, as well as two transmembrane domains, and one extracellular domain [194]. The N-terminal half of the protein is where the binding domains of CD36 exist. These binding domains are responsible for the binding of multiple ligands including thrombospondin, oxidized LDLs (ox-LDLs), and long chain and very long chain FAs [194, 195]. The extracellular

domain facilitates lipid binding, and the C-terminus can associate with Src-tyrosine kinases **(Figure 1.2)**.

The exact full structure of CD36 has yet to be fully delineated; however, several models have predicted the structure of CD36 with greater than 90% confidence [196, 197]. There is particular emphasis on modeling and identifying the exact structures involved in the FA binding pocket of CD36. A lysine residue at the 164 amino acid position (Lys-164) was found to be critical for FA binding [198]. It is suggested that this binding to Lys-164 by the FA further confers a conformational change within CD36 that facilitates cellular uptake [194, 199].

CD36 contains a central tunnel which traverses the entire length of the protein [194]. Models show that the FA binding pocket contains a groove which leads directly into the central tunnel of CD36 [194]. It is suggested that FA binding to Lys-164 in the binding pocket could displace a glutamate 335 residue at the top of the tunnel, allowing the FA access to the tunnel [198]. It was also discovered that the tunnel has an exterior entry point on the surface of CD36, separate from that of the FA binding pocket [198]. Free access to the tunnel by a FA may be restricted due to an acetylated Lys-231 residue at the tunnel entrance [198]. However, further studies beyond modeling are required to fully realize CD36's structure, particularly those structures of the FA binding pocket and the central tunnel.

The promoter region of CD36 contains peroxisome proliferator-activated receptor (PPAR) response elements (PPREs), and therefore PPARγ can regulate the mRNA transcription after binding to its ligands, of which include ox-LDLs and some prostaglandins [200]. Specifically, a positive feedback loop has been described in macrophages, where ox-LDLs absorbed by CD36 further active PPAR signaling through the mitogen-activated protein kinase (MAPK) signaling cascade, and thus increasing transcription of CD36, and in return further increasing ox-LDL uptake, leading to foam cell formation [201]. In addition to the PPREs, an interferon-γ-activated sequence (GAS) was also identified in the promoter region of CD36, of which can be bound by signal transducer and activator of transcription 3 (STAT3). Binding of STAT3 to this GAS region in response to several Janus kinase (JAK) ligands, such as epidermal growth factor (EGF), and promotes CD36 transcription [202].

CD36 is heavily post-translationally modified with modifications including phosphorylation, glycosylation, palmitoylation, acetylation and ubiquitination [194]. These modifications create a protein with a mass of approximately 88kD [203].Trafficking of the protein and membrane localization is heavily regulated. Specifically, there are two palmitoylation sites at each of the termini which are necessary for membrane localization. Furthermore, CD36 consists of several disulfide bridges within the carboxyl terminal half, which are also important for membrane association [194]. Moreover, CD36 contains nine extracellular sites where it is glycosylated (asparagine 79, 102, 134, 205, 220, 235, 247, 321, and

417) [204]. Glycosylation at each of these sites was shown to be critical for overall protein folding as well as cellular trafficking of the protein to the cell membrane [204].

CD36 may also be regulated based on fatty acid uptake and diet [194, 205]. A separate lysine residue within the FA binding pocket, Lys-166, was found to be acetylated which increases the hydrophobicity of the pocket, which is suggested in the regulation of FA access to the binding pocket or assist in the orientation of the FA once within the pocket [194]. Furthermore, CD36 may be nutritionally regulated via ubiquitination at two lysine residues within the C-terminus, which is upregulated with increased FFA availability and decreased with insulin treatment, suggesting a negative feedback loop during increased FFA availability [205].

1.11 CD36 Function

CD36 is expressed in various tissues throughout the body and is enriched in tissues of the circulatory system, immune cells, and particularly expressed in adipose tissues [206]. CD36 is also basally expressed at relatively lower levels in tissues of the digestive tract, particularly the small intestine and colon [206]. CD36 is a multifunctional glycoprotein and is a receptor for several ligands including thrombospondin (TSP-1), ox-LDLs and FAs and based on the tissues where CD36 is expressed, its function may be significantly different [207].

CD36 was first described as a receptor for TSP-1 and binding of TSP-1 by CD36 has been shown to regulate several cellular functions including the negative regulation of angiogenesis in ovarian tissues [208]. Furthermore, CD36 has been shown to facilitate the interaction between Src-homology-2-domain-containing protein tyrosine phosphatase (SHP-1) with vascular endothelial growth factor receptor 2 (VEGFR2) and, thus, reducing vascular endothelia growth factor signaling (VEGF) in endothelial cells [209]. CD36 binding to TSP-1 on the surface of platelets results in a hyperactive platelet state and promotes thrombosis [210]. CD36 can also facilitate intracellular signaling through other Src-tyrosine kinases in pathways including inflammation and atherosclerosis [210].

Aside from thrombospondin binding, more recently it has become well established that one of the major functions of CD36 is the binding and uptake of ox-LDLs and long-chain and very-long chain FAs [194, 211-213]. Interestingly, it was found that both FAs and ox-LDLs share the same binding position within the FA binding pocket, which include both the Lys-164 and Lys-166 residues [214]. The major areas of study delineating CD36's function in the binding of ox-LDLs involve its role in the progression of atherosclerosis [215]. Ox-LDLs are now recognized as a marker for inflammation and atherosclerosis, and CD36 mediated uptake of ox-LDLs has been directly linked to chronic inflammation and atherosclerosis [215]. More specifically, interactions between CD36 and ox-LDL can result in the formation of CD36-toll-like-receptor (CD36-TLR) complexes,

which activate nuclear factor kB (NF-kB) signaling and increased inflammation [216].

CD36 is highly expressed in adipose tissues and regulates lipid storage and lipolysis and in the presence of insulin, facilitates uptake of FAs [203, 215]. It was shown that the carboxyl group of a FA interacts directly with the Lys-164 at the entry position to the FA binding pocket, suggesting the Lys-164 aides in the proper positioning of the FA, orienting the long acyl chain of the FA so that it enters the pocket first [198]. Although the FA binding pocket is critical for the binding of FAs, it alone is not sufficient for full transfer of the FA into the cell. The central tunnel, which traverses the entire length of the protein, is connected to the FA binding pocket via a groove and is also critical for FA uptake as it is through this tunnel that FAs move through CD36 and to the cell membrane [194]. FAs bound and transported across the cell membrane by CD36 can then be used for a variety of purposes as described above, include as an energy source or substrates for membrane synthesis [215].

1.12 CD36 in Cancer

Due to the important role CD36 plays in the uptake of extracellular FAs, and the fact that dysregulated FA metabolism is a hallmark of cancer, it is possible that CD36 may play a role in the progression of CRC. CD36 has been shown to contribute to the progression and metastasis of hepatocellular and oral carcinoma, as well as glioblastoma, prostate, cervical, breast and ovarian cancers [149-152, 217-221].

In hepatocellular carcinoma (HCC), CD36 was shown to be significantly upregulated in patient tissues and established HCC cell lines [221, 222]. Furthermore, CD36 expression increased HCC cell proliferation, migration, and invasion *in vitro* as well as the promotion of tumor growth and metastasis *in vivo* [221]. Additionally, it was shown that CD36 promotes the progression of HCC by activating glycolysis through the upregulation of the Src/PI3K/AKT/mTOR signaling pathway [221]. Furthermore, it has also been shown that CD36 contributes significantly to epithelial to mesenchymal transition (EMT) in HCC via CD36 mediated FA uptake [222]. It was suggested that CD36 promotes this EMT in HCC through increased Wnt-TGFβ signaling; however more investigation is needed to fully discern the mechanisms behind CD36 -driven EMT [222].

As mentioned, aside from HCC, CD36 has also been shown to promote glioblastoma (GBM) progression [149]. Specifically, CD36 is highly upregulated in GBM cells and is enriched further in GBM cancer stem cells (CSCs) [149]. Furthermore, reduced expression of CD36 significantly inhibited the self-renewal and tumorigenic properties of GBM CSCs. Additionally, treatment of GBM CSCs with ox-LDLS enhanced cell proliferation [149]. Lastly, the expression of CD36 in GBM patients correlated with a poor prognosis [149]. However, exact mechanistic pathways in which CD36 enhances GBM progression have yet to be delineated.

CD36 has also been shown to promote the progression of ovarian, cervical and breast cancers [151, 218-220]. In ovarian cancer, it was shown that CD36 promotes an adipocyte-driven phenotype in ovarian cancer cells in a FA uptake dependent manner [151]. Additionally, inhibition or knockdown of CD36 completely attenuates this adipocyte dependent malignant phenotype as well as reduces cell adhesion, invasion, and migration *in vitro* [151]. Furthermore, human metastatic ovarian tumors exhibit an upregulation of CD36 expression and inhibition of CD36 via a neutralizing antibody reduced tumor burden *in vivo* [151].

CD36 is also highly expressed in cervical cancer tissues and data suggests that CD36 promotes cervical cancer cell proliferation, migration, and invasion both in vitro and *in vivo* [220]. Interestingly, TGFβ treatment of cervical cancer cells increased CD36 expression and downregulated the expression of e-cadherin, a crucial marker of EMT in several cancers [223]. Additionally, CD36 was also found to promote breast cancer progression and metastasis and is a poor prognostic factor for breast cancer patient survival [218, 219]. More specifically, breast cancer cell proliferation, which is enhanced via exogenous FA treatment, is significantly reduced in CD36 knockdown breast cancer cell lines [219]. FA treatment of these CD36 knockdown cell lines did not restore the more proliferative phenotype, suggesting that CD36 plays a significant role in breast cancer cell proliferation in a FA uptake dependent manner [219]. Furthermore, CD36 expression is higher in estrogen receptor rich (ER-rich) cell lines compare to ER-negative cells [218]. Specifically, CD36 promotes the expression of estrogen receptor alpha (ERα), which can be responsible for increased cell proliferation and apoptosis inhibition, as well as ERK1/2, which promotes breast cancer cell proliferation and invasion [224, 225]. Lastly, it was reported that CD36 contributes significantly to metastasis in oral carcinoma. CD36 high expressing oral carcinoma cells were shown to promote migration, invasion, and colony formation *in vitro* [150]. Furthermore, CD36 high expressing cells were shown to be unique in their ability to initiate metastasis in orthotopic oral carcinoma xenografts *in vivo* [150]. Inhibition of CD36 via treatment with a neutralizing antibody completely abolished local invasion and metastasis of oral carcinoma tumors *in vivo* [150]. Interestingly, inhibition and knockdown of CD36 appeared to have no significant effect on primary oral carcinoma tumor growth, and only attenuated metastatic disease [150].

Together, these data suggest that CD36 plays an important role in the progression and metastasis of many cancers. However, further investigation is critical to understand the mechanistic pathways involving CD36's pro tumorigenic and metastatic characteristics. Moreover, no previous studies have investigated the relationship of CD36 driven FA metabolism and that of *de novo* FA synthesis.

1.12.1 CD36 as a Chemotherapeutic Target

CD36 has been the subject of a few pre-clinical and phase I/II clinical trials in renal cell carcinoma, non-small cell lung cancer, and soft tissue sarcoma [226].

Interestingly, each of these clinical trials have targeted CD36 from the perspective of its binding to TSP-1 [226]. Due to previous data suggesting that binding of TSP-1 by CD36 may upregulate anti-angiogenesis, these multiple clinical trials involved TSP-1 mimetic peptides, which further enhance TSP-1-CD36 downstream signaling as described previously [208, 209, 226]. These drugs include ABT-510, ABT526, and ABT-898 as well as CVX-22, and CVX-045 [227, 228]. The ABT drugs showed promise in pre-clinical *in vivo* studies but failed in phase II clinical trials after results showed the drug had no significant effect on tumor progression and patients displayed severe side effects [228]. Likewise, the CVX drugs showed significant tumor regression in animal studies, but did not make it past phase I clinical trials due to adverse side effects and poor response rates [227]. However, no such clinical trials targeting CD36 in CRC have previously been explored.

Due to the poor efficacy of targeting CD36 via TSP-1 binding, alternate pathways in which CD36 is involved may be more viable. Since recent studies have established CD36 as a prominent FA transporter, and since altered FA metabolism is a hallmark of cancer, targeting the FA transport function of CD36 could be a possible therapeutic strategy [128, 194, 229]. Furthermore, more investigation into the relationship between CD36 mediated FA uptake and *de novo* FA synthesis may yield more potential therapeutic modalities for the treatment of CRC.

1.13 Hypothesis

The purpose of this project was to understand whether CD36 contributes to CRC progression and metastasis as well as what relationship CD36 may have with *de novo* FA synthesis. The hypothesis is that: (1) Inhibition of FASN via TVB inhibitors upregulates CD36 and, thus, exogenous FA uptake, (2) CD36 upregulation promotes CRC primary tumor proliferation and survival, and (3) CD36 further enhances CRC invasion and metastasis.

Table 1.1 CRC survival rates by stage.

Colon Cancer Stage	5-Year Survival Rate
Stage I	92%
Stage IIA	87%
Stage IIB	63%
Stage IIIA	83%
Stage IIIB	64%
Stage IIIC	44%
Stage IV	11%

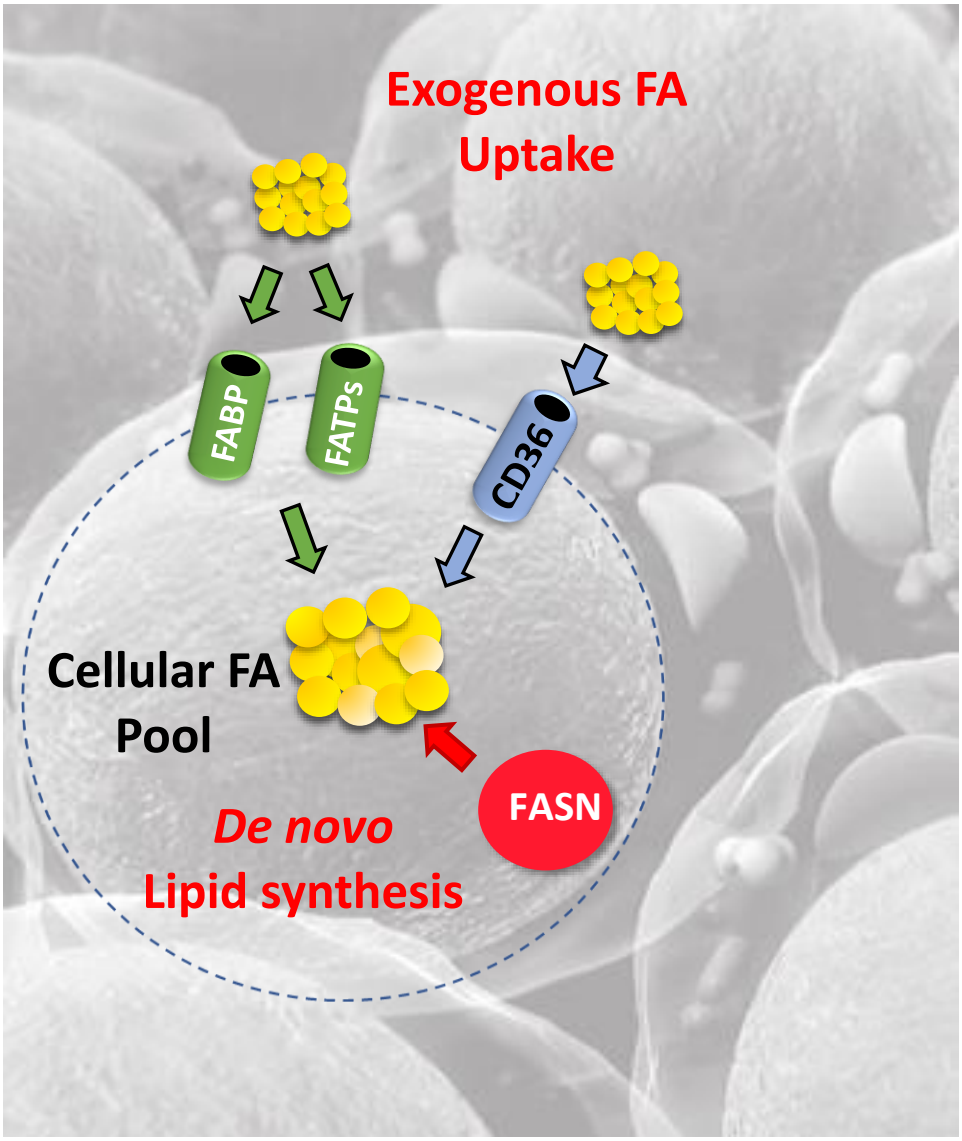


Figure 1.1 Diagram of cellular FA acquisition. Cell can uptake exogenous FAs through FA transporters which include the FABPs, FATPs, and CD36 or they can synthesize them via *de novo* lipid synthesis which involves the multi-enzyme FASN.

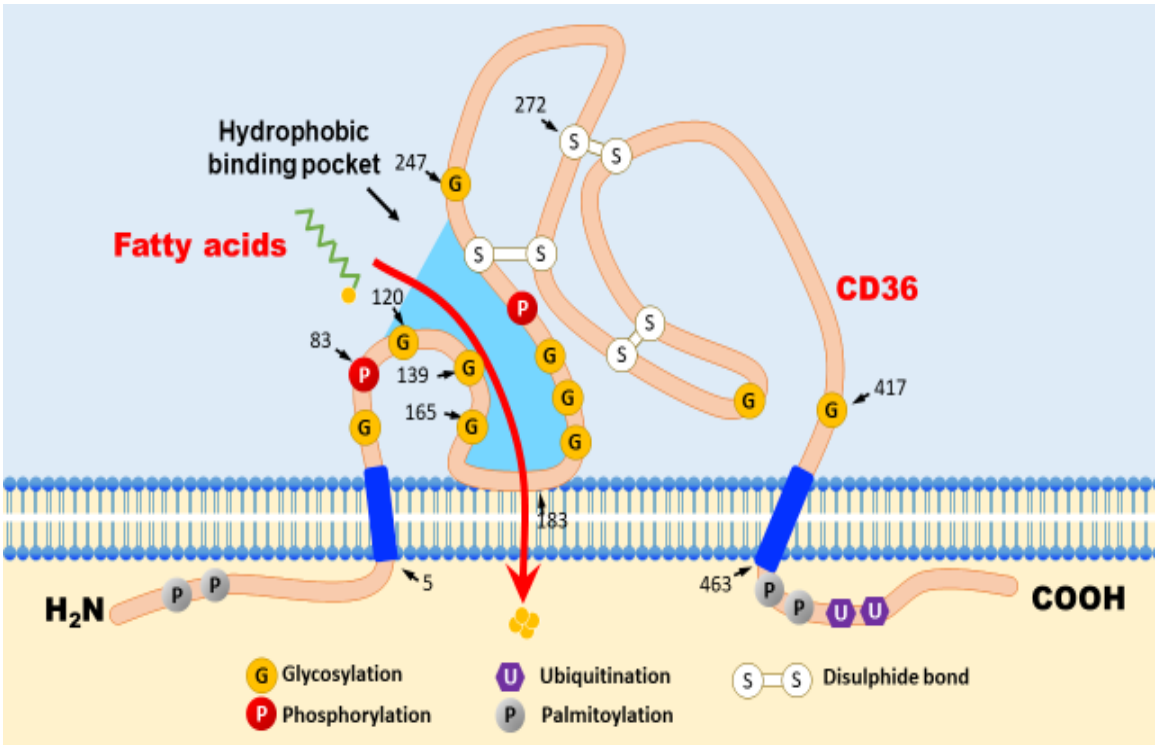


Figure 1.2 Structure of CD36. Schematic showing the various post translational modifications observed with CD36 and highlighting the extracellular FA binding pocket.

CHAPTER 2. INHIBITION OF FATTY ACID SYNTHASE UPREGULATES EXPRESSION OF CD36 TO SUSTAIN PROLIFERATION OF COLORECTAL CANCER CELLS

2.1 Abstract

Purpose: Altered FA metabolism is a hallmark of cancer and a potential target for cancer therapy. FA Translocase (CD36) has an important role in FA metabolism as a FA transporter. FA Synthase (FASN), a key enzyme of de novo lipogenesis, is associated with poorer prognosis in many cancers including colorectal cancer (CRC). However, the role of CD36 in CRC as well as its relation to FA synthesis is not understood. The purpose of our study was to: (i) determine the functional importance of CD36 in CRC and (ii) investigate the interconnection between CD36 and FASN expression in CRC cells.

Methods: CD36 and FASN expression was assessed in a CRC tumor microarray (matched normal colon and primary tumor; 56 cases) as well as matched normal colon, primary and metastatic tumors by immunohistochemistry. Cellular proliferation was assessed in control and CD36 shRNA knockdown CRC cells and in primary CRC cells established from patient-derived xenografts treated with Sulfo-N-succinimidyl oleate (SSO), a CD36 inhibitor, and TVB-3664, a FASN inhibitor currently in clinical trial. The effect of CD36 knockdown on tumor growth was assessed in vitro using a colony formation assay and in vivo using a subcutaneous xenograft model. Expression of pro-survival and apoptotic markers was assessed in CD36 knockdown and overexpression CRC cells and in Pt 2402 CD36high and CD36low isogenic cells via western blot. CD36 localization was assessed via confocal imaging. Effect of FASN knockout on CD36 expression in a genetically modified mouse model was assessed via qRT-PCR and western blot.

Results: CD36 is overexpressed in CRC primary tumors compared to normal colon mucosa. Pharmacological inhibition of FASN and shRNA-mediated knockdown of FASN specifically induces CD36 expression and its translocation to the plasma membrane in CRC cells and human tissues. Furthermore, deletion of FASN in APC/Cre mice significantly increases CD36 mRNA and protein expression. Additionally, knockdown of FASN increase extracellular FA uptake in a CD36 dependent manner. Cellular proliferation is significantly reduced when CD36 is inhibited by SSO. Inhibition of CD36 reduces CRC cell proliferation and colony formation *in vitro*. Knockdown and chemical inhibition of CD36 decreased expression of survivin, which has been shown to promote cancer cell survival in many tumor types. Overexpression of CD36 increased survivin expression in CRC cells. A higher level of survivin was observed in Pt 2402 $CD36^{high}$ cells as compared to Pt2402 $CD36^{low}$ cells. Knockdown and pharmacological inhibition of CD36 significantly reduces the ability of CRC cells to form xenograft tumors in vivo. Further reduction on CRC cell proliferation *in vitro* is observed when SSO treatment is combined with TVB-3664.

Conclusions: This study for the first time demonstrates that inhibition of FASN selectively upregulates CD36 in multiple colorectal cancer models including human tissues, colorectal cancer cells with shRNA mediated knockdown of FA synthase and genetically modified mouse tissues with heterozygous and homozygous deletion of FASN. In summary, our study suggests that upregulation of CD36 expression is a potential compensatory mechanism for FASN inhibition and that inhibition of CD36 can improve the efficacy of FASN-targeted therapy.

2.2 Introduction

Colorectal cancer (CRC) is the leading cause of non-smoking related cancer deaths in the world [1]. Altered FA metabolism is a hallmark of cancer and a potential target for therapeutic intervention [128-130]. FA Synthase (FASN), a key enzyme of *de novo* lipogenesis, is significantly upregulated in CRC and promotes tumor growth and metastasis [153, 230, 231]. Novel FASN inhibitors developed by Sagimet Biosciences show anti-cancer activity in lung, prostate, ovarian and colon cancer models *in vitro* and *in vivo* [185, 232, 233], and are currently being tested in phase I/II clinical trials [234-236]. Our studies show anti-tumor activity of TVB inhibitors in primary CRC cells and CRC patient-derived xenograft (PDX) models [185, 237].

While most tumors exhibit a shift toward FA synthesis, they can also scavenge lipids from their environment [130]. FA Translocase (CD36), a multifunctional glycoprotein, has an important role in FA metabolism as a FA receptor and transporter [194, 238]. CD36 translocate to the plasma membrane, where an extracellular domain of the protein binds low density lipoproteins and transports them across the plasma membrane into the cytosol, thus playing a critical role in the metabolism of extracellular FAs [194, 238, 239]. CD36 is subject to various types of post-translational modifications. Glycosylation, ubiquitination and palmitoylation are involved in regulating CD36 stability and the rate of FA uptake [240]. Recent studies have shown that CD36 is highly expressed and enhances the progression of solid malignancies such as breast, ovarian, gastric, and glioblastoma cancers [149, 151, 241, 242]. Silencing CD36 in human prostate cancer cells reduces FA uptake and cellular proliferation [243]. Furthermore, the presence of CD36 positive, metastasis initiating cells correlates with a poorer prognosis in glioblastoma and oral carcinoma [149, 150]. The contribution of CD36 to CRC progression has not yet been investigated.

Since cancer cells utilize both endogenously-synthesized lipids and exogeneous FAs [244], and our published data indicate that an enhanced uptake of dietary FAs may be a potential mechanism of resistance to FASN inhibitors [185], the goal of this study was to evaluate the interconnection between these two pathways.

We found that CD36 is significantly overexpressed in CRC and that there is a correlation between expression of FASN and CD36 in primary human CRC

specimens. We demonstrate that a decrease in FASN expression is associated with selective induction of CD36 and that this phenomenon is consistent among multiple cancer models. Pharmacological and shRNA-mediated inhibition of CD36 decreases proliferation of primary CRC cells *in vitro* and inhibits tumor growth *in vivo*. We also show that CD36 overexpression is associated with upregulation of survivin, a protein linked to apoptosis resistance, metastasis, bypass of cell cycle checkpoints, and resistance to therapy [245, 246]. Consistent with our *in vitro* data, we show that $CD36^{high}$-expressing cells, isolated from CRC PDXs, have a significantly higher level of survivin as compared to $CD36^{low}$-expressing cells from the same tumor. Our results also demonstrate that combined inhibition of FASN and CD36 has a synergetic effect on inhibition of cellular proliferation suggesting that combination treatment may be a potential therapeutic strategy for CRC.

Together, our findings demonstrate the tightly regulated interconnection between *de novo* lipid synthesis and CD36-mediated lipid uptake in CRC progression during targeted inhibition of FASN, suggesting that inhibition of CD36 may be necessary to improve the efficacy of FASN-targeted therapy.

2.3 Results

2.3.1 CD36 Protein is Overexpressed in CRC

Upregulation of lipid metabolism is a common characteristic of many solid malignancies, and frequently, enhanced *de novo* lipogenesis occurs concomitantly with enhanced import of lipids from the extracellular space [129, 247]. In our previously published study we showed that FASN is significantly overexpressed in primary tumor tissues as compared to matched normal colon mucosa using tissue microarray analysis (TMA) [248]. Using the same TMA, we assessed the expression of CD36 levels in tumor tissues and found that expression was significantly higher compared to normal colon mucosa as determined by statistical evaluation of immunoreactivity scores. We noted that the expression of CD36 is predominantly cytosolic in primary CRC tumors (**Figure 2.1 A-B**). Interestingly, statistical analysis via Spearman Correlation showed a positive correlation between expression of CD36 and FASN in primary CRC tumor tissues, but it was not statistically significant (Spearman r =0.21743, n = 56). We have also detected an increase in expression of CD36 in CRC metastasis to liver and lung (**Figure 2.1 C**).

Using the Cancer Genome Atlas (TCGA) , we also analyzed FASN and CD36 mRNA expression. Consistent with protein data, the level of FASN mRNA is significantly higher in tumor tissues as compared to normal mucosa (**Supplementary Figure S2.1 A**). In contrast, we found that the level of CD36 mRNA is significantly lower in cancer tissues as compared to normal tissues (**Supplementary Figure S2.1 B**). Interestingly, according to data analysis from the Human Protein Atlas, the high mRNA expression of CD36 (n = 131) is associated with poor prognosis in CRC with 5-year survival of 53% patients as compared to

5-year survival of 64 % patients with low CD36 mRNA expression (n = 466) (https://www.proteinatlas.org/ENSG00000135218-CD36/pathology/colorectal+cancer). Statistical analysis of correlation between FASN and CD36 revealed a significant negative correlation between FASN and CD36 mRNA levels in tumor tissues, but not in normal tissues (**Figure 2.1 D**).

To further delineate association between expression of FASN and CD36, we analyzed expression of these proteins in fresh human normal colon mucosa, primary CRC tissues and metastasis (**Figure 2.1 E-F**). The predicted molecular mass of CD36 protein is 53kD. However, due to the post-transcriptional modifications including extensive protein glycosylation, it is widely reported as approximately 80- 88kD protein [238, 240, 249]. This 80-88kD size will be shown for all *in vitro* and *in vivo* data figures of this manuscript. In the analyzed tissues sample set, the expression of FASN is higher in primary tumors as compared to normal mucosa in most cases. Due to FASN being expressed in healthy liver tissue, it is not surprising to see that its expression is higher in the normal liver as compared to liver metastasis. CD36 expression seems to be higher or the same in primary tumors as compared to normal colon mucosa. However, expression of CD36, particularly its glycosylated form, is much higher in liver metastasis as compared to normal liver or normal colon mucosa (**Figure 2.1 E**).

To further analyze CD36 in colorectal cancer we used several PDX models, which retain the intratumorally clonal heterogeneity and tumor microenvironment of the parent tumor through passages in mice [185, 250]. We analyzed the expression of CD36 in nine PDXs established from primary tumors and CRC metastasis [185], and found that CD36 (88kD) is mostly associated with PDX established from metastatic tumors with an exception of Pt 2568 which was established from primary CRC tumor [185] (Supplementary **Figure S2.1 C**).
Together, these data demonstrate that CD36 is upregulated and exhibit multiple post-translational modifications in CRC and that there is a significant inverse correlation between mRNA expression of FASN and CD36 in primary human CRC.

2.3.2 FASN Selectively Regulates Expression of CD36

To test whether alterations in FASN expression affect FA uptake, we assessed the expression of major FA transporters (FATPs and CD36) in HCT116 NTC and FASN shRNA CRC cells and found that FASN selectively upregulates mRNA expression of CD36, but not other FAs transporters (**Figure 2.2 A**). To confirm that FASN selectively upregulates CD36, we treated fresh CRC human tissue slices with TVB-3664 and assessed the expression of FA transporters, including CD36. Consistent with our *in vitro* data, in all three CRC cases (**Supplementary Table S2.1**), we observed that CD36 mRNA expression increased at least two-fold and as much as four-fold when tissues were treated with TVB-3664. No changes were observed in expression of the other FA transporters tested (**Figure 2.2 B**).

To further elucidate whether the level of endogenous FA synthesis affects the expression of CD36, we next treated primary CRC cells from Pt 93 and Pt 130 with TVB-3664 for six days at a concentration of 0.2μM as previously described [185]. Inhibition of FASN led to an increase in CD36 mRNA and protein expression in both cell lines (**Figure 2.2 C**). Consistently, shRNA-mediated knockdown of FASN in HCT116 and HT29 cell lines led to an increase in CD36 expression in normal and hypoxic conditions in both cell lines (**Figure 2.2 D, Supplementary Figure S2.2 A**). Interestingly, shRNA-mediated knockdown of CD36 does not affect FASN expression, suggesting a one-dimensional relationship between the two proteins (**Supplementary Figure S2.2 B**).

The adenomatous polyposis coli (APC) gene product is mutated in the vast majority of human CRC and deletion of the APC gene leads to intestinal tumor formation in mice [251]. In agreement with in vitro data, the analysis of intestinal tumors from mice with hetero- and homozygous deletions of FASN on C57BL/6-Apc/Cre background showed that deletion of FASN significantly upregulates CD36 expression (**Figure 2.2 E-F**). Collectively, these data suggest that inhibition of FASN leads to selective upregulation of CD36 expression.

2.3.3 Inhibition of FASN Leads to CD36 Translocation to Plasma Membrane

Confocal imaging of primary Pt 93 CRC cells, control and treated with TVB-3664, shows that CD36 protein expression is upregulated and primarily localized to the plasma membrane when FASN is inhibited by TVB-3664 (**Figure 2.2 G**). To confirm these data, primary CRC cells from Pt 93 and Pt 130 were treated with 0.2 μM TVB-3664 for six days in normal or serum-starved conditions and labeled with CD36-FITC antibody. Flow cytometry analysis was performed; the results confirmed that inhibition of FASN activity by TVB-3664 led to an increase in membrane-associated CD36 when compared to control cells in both cell lines in normal and serum-starved conditions (**Figure 2.2 H**).

To test whether this upregulation and translocation of CD36 to the plasma membrane was related to FA metabolism, a FA uptake assay was performed. HCT116, NTC and FASN shRNA cells were plated and treated with BODIPY FL and imaged using confocal microscopy. We observed that FASN knockdown increases FA uptake as indicated by an increase in BODIPY FL staining (**Figure 2.2 I**). Furthermore, to test that this increase in FAs within the cell was due to CD36 upregulation, we treated NTC and FASN shRNA cells with neutralizing antibody for CD36. Blocking CD36 has a minimum effect in NTC cells, but significantly decreases BODIPY FL uptake in FASN shRNA cells, further confirming that inhibition of FASN increases FA uptake via upregulation of CD36 (**Figure 2.2 I**).

2.3.4 Inhibition of CD36 reduces CRC Cell Proliferation *in vitro*

We have previously shown that stable knockdown and pharmacological inhibition of FASN are associated with a decrease in cellular proliferation and

tumor growth [153, 185]. However, the observed effects *in vivo* were not as prominent as the effects *in vitro*, suggesting the potential compensatory effects of diet and exogeneous FA uptake on tumor growth [153, 185]. To test whether blocking fatty-acid uptake via CD36 has an effect on CRC cell proliferation, primary CRC cells, Pt 93 and Pt 130, were treated with the chemical CD36 inhibitor sulfosuccinimidyl oleate (SSO), which binds to CD36 via Lys164 in the hydrophobic cavity thereby impairing CD36-mediated FA uptake [240, 252], at 100μM in both normal and serum free medium (SFM) conditions. Under both conditions, primary CRC cells treated with SSO exhibited decreased cellular proliferation. Interestingly, sensitivity of both cell lines to SSO increased in SFM (**Figure 2.3 A**). To evaluate differences in CD36 expression in normal and SFM, we performed confocal microscopy on Pt 93 cells cultured in normal and SFM conditions. We found that starvation of CRC cells leads to upregulation of CD36, which could explain an increase in sensitivity to SSO treatment (**Figure 2.3 B**).

To assess the effect of CD36 overexpression on apoptotic markers we performed an Apoptosis Antibody Array. Data showed that overexpression of CD36 decreased caspase-3 cleavage and increased expression of survivin, a protein overexpressed in most transformed cell lines and malignancies and associated with poor clinical outcome (**Supplementary Figure S2.3 A**) [246, 253]. Consistently, western blot analysis of control and SSO-treated Pt 130 and Pt 93 primary CRC cells showed an increase in cleaved caspase-3 in both cell lines. A decrease in expression of survivin was observed in Pt 130 cells only (**Supplementary Figure S2.3 B**). Consistent with pharmacological inhibition of CD36, shRNA-mediated knockdown of CD36 lead to a significant decrease in cellular proliferation and expression of survivin and pAkt in HCT116 cells (**Figure 2.3 C-E**). Furthermore, shRNA-mediated knockdown of CD36 inhibits colony formation in the HT29 cell line (**Supplementary Figure S2.3 C**). Together, these data demonstrate that CD36 promotes cellular proliferation in CRC.

2.3.5 Inhibition and Knockdown of CD36 Reduces Xenograft Tumor Growth *in vivo*

To further investigate the role of CD36 in CRC tumor growth, HCT116 subcutaneous xenografts were treated with vehicle or SSO daily for 5 weeks. SSO treatment lead to significant decreases in tumor volume compared to vehicle control with no observable SSO toxicity as indicated by unchanged animal weight (**Figure 24 A**). Furthermore, consistent with *in vitro* data, analysis of tumor tissues treated with SSO show a decrease in survivin mRNA (**Figure 2.4 B**). FASN mRNA expression does not change with inhibition of CD36, further supporting the notion of a one directional relationship between the two proteins. Interestingly, SSO treatment led to an increase in CD36 mRNA suggesting that the potential compensation for the lack of functional CD36 was due to antagonistic action of SSO (**Figure 2.4 B**).

To further elucidate the role of CD36 in CRC tumor growth, the CRC cell lines, HCT116, HT29 and HT29 LuM3 (an HT29 cell line that was trained to

efficiently metastasize to lung via *in vivo* selection process [254]), were established as subcutaneous xenografts. Interestingly, *in vivo* selection led to an increase in CD36 expression in HT29 LuM3 as compared to parental HT29 cells (**Supplementary Figure S2.4 A**). HCT116, HT29 and HT29 LuM3 cells (NTC and shRNA mediated CD36 knockdown cell lines) were injected subcutaneously into Nu/Nu mice and tumor growth was measured. Knockdown of CD36 in HCT116 cells markedly attenuated the growth of xenograft tumors compared to NTC (**Figure 2.4 C, Supplementary S2.4 B**). In the case of CD36 shRNA #2 and CD36 shRNA #4 cells, we were able to identify microtumors at the site of injections. Tumor tissues were stained for Ki67, a known marker for tumor cell proliferation and growth [255]. Ki67 expression was greatly reduced in CD36 knockdown tumors as compared to control (**Figure 2.4 D**). In contrast to HCT116 cells, CD36 knockdown in HT29 did not significantly affect tumor growth, suggesting that this cell line may not be dependent on CD36 due to considerably lower CD36 expression as compared to HCT116 cells (**Supplementary Figure S2.4 A, C, D**). However, CD36 knockdown using CD36 shRNA #4 in HT29 LuM3 cells, which have higher levels of CD36 expression as well as higher metastatic potential [254] (**Supplementary Figure S2.4 A**), lead to a more prominent inhibition of tumor growth and a decrease in tumor weight (**Figure 2.4 E**). Moreover, consistent with our *in vitro* data, qRT-PCR analysis of tumor tissues demonstrates a decrease in survivin expression when CD36 is knocked down in HT29 LuM3 tumors (**Figure 2.4 F**). Thus, these data further support the role of CD36 in promoting CRC tumor growth.

2.3.6 High Expression of CD36 is Associated with an Increase in Survivin in CRC

To further establish that CD36 promotes cellular proliferation via upregulation of pro-survival pathways, we utilized a PDX tumor model, Pt 2402, which was established from a CRC metastasis to the lung [185] and is positive for CD36 expression (see **Figure 2.1 E**). Tumor tissue from 1st generation Pt 2402 PDX was inoculated into NOD/SCID mice and grown to approximately 1cm^3 volume. The tumor was excised, digested as previously described to a single cell suspension [248], stained with CD36-FITC and sorted via flow cytometry. The top 10% of the brightest green fluorescent protein (GFP) positive cells (117,000 cells), designated CD36^{high}, and the bottom 10% of GFP negative cells (3,120,000 cells), designated CD36^{low}, were sorted separately, placed in Matrigel, and sequentially implanted into NOD/SCID mice and allowed to grow. The tumor established from CD36^{high} cells grew much larger compared to the CD36^{low} tumor (tumor volume 2419.64mm^3 versus 53.57mm^3, respectively) (**Figure 2.5 A-B**). Western blot analysis of tumor tissues from CD36^{high} and CD36^{low} cells showed an increase in survivin expression in the CD36^{high} tumors in comparison to the CD36^{low} tumors (**Figure 2.5 C**). Interestingly, similar to our data obtained from TMA analysis, we observed that FASN was higher in CD36^{high} cells as compared to CD36^{low} cells, further supporting a potential interconnection between these two proteins (**Figure 2.5 C**). Ki67 staining of Pt 2402 CD36^{high} and CD36^{low} tumors showed a significant

reduction in Ki67 expression in the $CD36^{low}$ tumors compared to $CD36^{high}$ (**Figure 2.5 D**).

To confirm that an increase in CD36 expression is associated with an increase in survivin expression, we overexpressed CD36 in the established HCT116 CRC cell line. Western blot analysis of HCT116 cells demonstrated CD36 overexpression leads to an increase in expression of survivin and activation of Akt, an upstream translational regulator of surviving in CRC [256], as well as a decrease in cleaved-PARP (**Figure 2.5 E**). Therefore, taken together, our data suggest that upregulation of pAkt and survivin are potential mechanisms by which CD36 promotes CRC cell proliferation and tumor growth.

2.3.7 Inhibition of FASN and CD36 in Combination Reduce Primary CRC Proliferation in vitro

Both *de novo* synthesized and exogenous FA play important roles in carcinogenesis [247, 257], To extend our findings that FASN inhibition upregulates the expression of CD36 and to further test whether inhibition of CD36 can improve the efficacy of TVB-3664, primary CRC cells from Pt 93 and Pt 130 were treated with CD36 inhibitor SSO and FASN inhibitor TVB-3664, alone or in combination, in both normal and serum-starved media. Cellular proliferation was significantly reduced in both SSO- and TVB-3664-treated cells and was further significantly reduced in cells that received combination treatment (**Figure 2.6 A**). Western blot analysis of CRC cells treated with a combination of TVB-3664 and SSO shows that combination treatment significantly reduces expression of survivin in PT130 and HCT116 cell lines but not in Pt93 cell line as compared to control or single agent treatment alone (**Figure 2.6 B**). Combination treatment was also associated with reduced expression of cyclin D1 in Pt93 and HCT116 cell lines. Interestingly, cyclin D1 in Pt130 cells increased expression in combination treatments. This suggests a different mode of action and sensitivity to SSO and TVB-3664 in Pt130 when compared to other CRC cell lines. Collectively, these data suggest that inhibition of both FA synthesis and FA uptake may be a potential therapeutic strategy for CRC. However, further studies are necessary to evaluate the effect of combinational treatment *in vivo.*

2.4 Discussion

Our previous studies demonstrate that the effect of FASN inhibition on cellular proliferation *in vitro* does not always translate to the same effect on tumor growth *in vivo* [153, 185]. Despite a significant decrease in cellular proliferation in primary CRC cell lines treated with TVB-3664, the efficacy of TVB-3664 in PDX models was much lower, suggesting a potential compensatory impact of diet on the effect of FASN inhibitors [185, 237]. Therefore, the goal of this study was to

delineate the effect of FASN inhibition on exogeneous FA uptake and elucidate the effect of FA uptake on sustaining cellular proliferation.

Here, for the first time, we report that inhibition of FASN leads to a selective upregulation of CD36 expression. CD36 enhances FA uptake and FA oxidation and plays a critical role in cancer cell growth and metastasis [149-151, 258, 259]. Consistent with reports that CD36 is upregulated in breast cancer and glioblastoma [260], we found that CD36 is highly expressed in CRC as compared to normal mucosa. Tumor stroma is deficient for CD36 expression [226]. High stromal content in fresh primary CRC tumors can potentially explain why fresh CRC tissue analysis shows inconsistent result for CD36 expression in primary CRC as compared to normal colon. Interestingly, based on tissue analysis, we found that high expression of CD36 is primarily associated with CRC metastasis suggesting that metastatic tumors are more dependent on FA uptake as compared to primary CRC. Our findings are supported by multiple studies showing CD36 involvement in metastatic disease [150, 261, 262]. Interestingly, even though we have identified a positive correlation between the protein expression of FASN and CD36 using TMA analysis, based on TCGA data, there is a significant inverse correlation between these two proteins at the mRNA levels. Indeed, we found that inhibition of FASN selectively upregulates CD36 mRNA and protein expression, but not expression of other FA transporters in multiple models including CRC cells, tumor xenografts, genetically modified mice and in human tissues. Interestingly, we did not note any significant changes in FASN expression when the expression of CD36 was altered, suggesting that the level of *de novo* lipid synthesis is not regulated by FA uptake via CD36 in our models. Current understanding of the regulation of CD36 expression is rather limited [263], and how CD36 expression is regulated in cancer, and in particular in CRC, is not known. Several transcriptional activators have been implicated in regulation of CD36 expression including peroxisome proliferator-activated receptors (PPARs), CCAAT/enhancer-binding protein and HIF-1 [238]. Ongoing studies in our laboratory are investigating CD36 expression in different cell types in primary and metastatic CRC and potential mechanisms of CD36 regulation by FASN.

It has been reported that siRNA-mediated inhibition of CD36 decreases cellular proliferation in MCF-7 breast cancer cells [241]. Additionally, CD36 has pro-tumorigenic and progression properties in glioblastoma stem cells [149]. In agreement with these data, our study shows that chemical inhibition and stable knockdown of CD36 via shRNA in established and primary CRC cells decrease cellular proliferation. Consistent with data using a specific small molecule CD36 inhibitor, 2-methylthio-1,4-napthoquinone (MTN), in glioblastoma stem cells [149], inhibition of CD36 with SSO is associated with a decrease in activation of pAkt. We have also showed that CD36 regulates survivin, a member of the inhibitor of apoptosis (IAP) family that is highly expressed in most cancer and associated with a poor prognosis [264]. The pro-survival role of CD36 in CRC is further supported by data showing that Pt 2402 $CD36^{high}$ cells have a much higher propensity to

establish xenograft tumors, which grow significantly faster and express higher levels of survivin, in comparison to $CD36^{low}$ cells.

Interestingly, in a previously published study in oral carcinoma, the effect of CD36 inhibition was associated with inhibition of metastasis, but not with growth of primary oral cancers [150]. In contrast to these findings, our study suggests a critical role of CD36 in CRC proliferation and tumor growth *in vivo* with both chemical inhibition via SSO as well as shRNA-mediated knockdown of CD36 in xenografts using multiple established cell lines.

Novel FASN inhibitors, TVBs, have demonstrated anticancer activity in multiple preclinical models [129], and TVB-2640 is currently in a number of clinical trials, including one at the University of Kentucky's Markey Cancer Center (https://www.cancer.gov/about-cancer/treatment/clinical-trials/search/v?id=NCI-2016-01710&r=1). Thus, it is crucial to identify and understand potential resistance mechanisms to FASN-targeted therapy. The current study demonstrates that inhibition of FASN leads to upregulation of CD36 expression and its translocation to the plasma membrane. One of the primary roles of CD36, when located within the cell membrane, is the transport of FAs [194, 238]. Therefore, this upregulation of membrane bound CD36 and, consequently, an increase in FA uptake, could be a potential mechanism of resistance to FASN inhibition. Importantly, our data demonstrate that the combined inhibition of CD36 and FASN has a synergistic effect on inhibition of cellular proliferation as well as survivin and cyclin D1, further suggests that targeting FA uptake may be a potential therapeutic approach to increase the efficacy of FASN inhibitors.

We have previously reported that the level of FASN expression determines the sensitivity of tumors to TVB compounds [185]. Consistently throughout this study, we observed that higher expression of CD36 in HT29 LuM3 cells [254], as compared to parental HT29 cells, makes these cells more sensitive to CD36 inhibition via CD36 shRNA and increases xenograft tumor growth inhibition compared to HT29 xenografts. Furthermore, the mutational and metabolic profiles of tumors determine tumor cell response to multiple therapies including metabolic inhibitors [265, 266]. Different genetic profiles and metabolic features can explain the varying levels of response to FASN and CD36 inhibition. Pt 93 and Pt130 cells have KRAS and V600E BRAF mutations. Pt 130 also carries an FGFR mutation [185]. The HCT116 cell line is a KRAS mutant, but BRAF wild type, compared to HT29 which has a V600E BRAF mutation but KRAS wild type [267]. Furthermore, TVB-3664 seems to have more efficacy in activating PARP cleavage compared to SSO, suggesting that the inhibition of lipid synthesis leads to activation of apoptosis through distinct pathways other than those related to the inhibition of exogenous FA uptake. Our ongoing studies in the laboratory are focused on identifying the mutational and metabolic features of tumors that would determine their sensitivity to lipid metabolism targeted therapies.

2.5 Conclusions

Multiple studies suggest that FA metabolism in adipose tissue is a major contributor to the etiology of obesity and diabetes [268]. Obesity is associated with chronic elevation of free FAs (FFA) within the blood serum, which promote insulin resistance and contribute to the development of systemic hyperglycemia [269]. Interestingly, FASN expression is directly linked to obesity and type 2 diabetes [270] and CD36 protein expression is upregulated in both obese patients and type 2 diabetics [271]. Therefore, the findings from this study support the idea that targeting both FASN and CD36 in combination may have therapeutic potential not only in cancer but also in metabolic disorders such as obesity and diabetes.

This report is the first to describe the functional importance of CD36 and its indolent role in FA metabolism in the setting of CRC. It is also the first to describe the interconnection between FASN and CD36 and provides a strong rationale for further investigation into the interconnection of *de novo* lipogenesis and FA uptake that could potentially lead to the development of new therapeutic strategies for CRC and other solid malignancies as well as, potentially, in some metabolic disorders.

2.6 Citation

Drury, J., Rychahou, P. G., He, D., Jafari, N., Wang, C., Lee, E. Y., Weiss, H. L., Evers, B. M., & Zaytseva, Y. Y. (2020). Inhibition of Fatty Acid Synthase Upregulates Expression of CD36 to Sustain Proliferation of Colorectal Cancer Cells. *Frontiers in oncology*, *10*, 1185. https://doi.org/10.3389/fonc.2020.01185

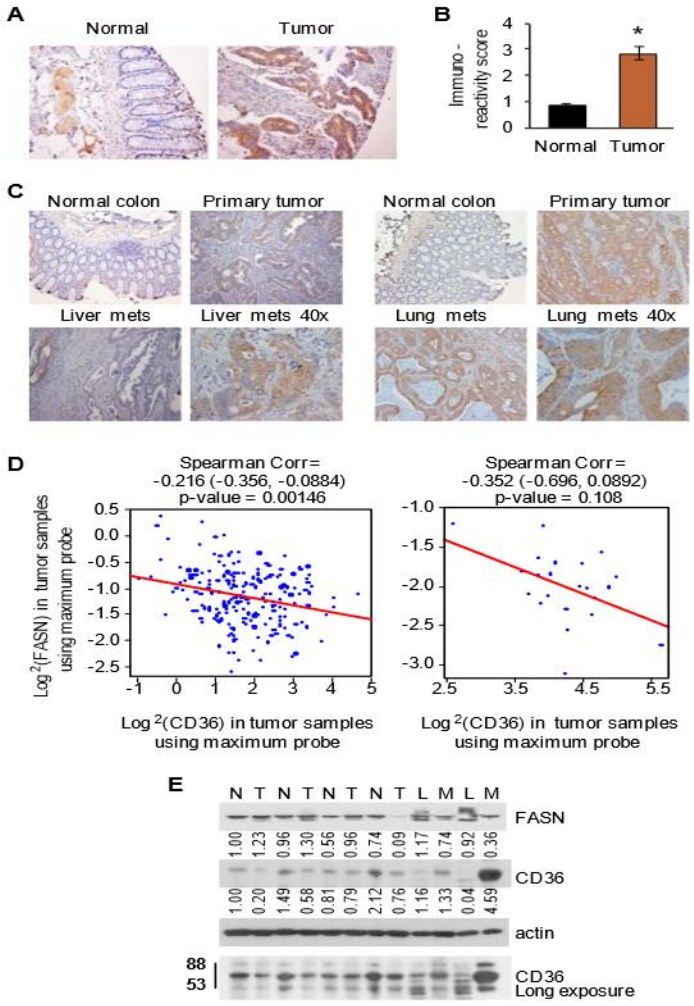


Figure 2.1 CD36 is overexpressed in human CRC.
(A-B) Immunoreactivity score of CD36 expression was analyzed in matched normal colon mucosa and tumor tissues from patients diagnosed with Stage I-IV CRC (TMA: n=56, *p<0.001 vs. normal tissue). **(C)** CD36 staining in matched normal colon mucosa, primary CRC and CRC metastasis to liver and lung (representative images are shown; liver [n=12] and lung metastasis [n=5]) **(D).** Correlations between FASN and CD36 was determined based on RNASeq data of CRC patient samples (n=22 of normal tissues and n=215 of tumors) from The Cancer Genome Atlas. **(E)** Expression of FASN and CD36 in human normal colon mucosa and tumor tissues. N-normal mucosa, T-primary tumor, L- normal liver tissue, M-liver metastasis.

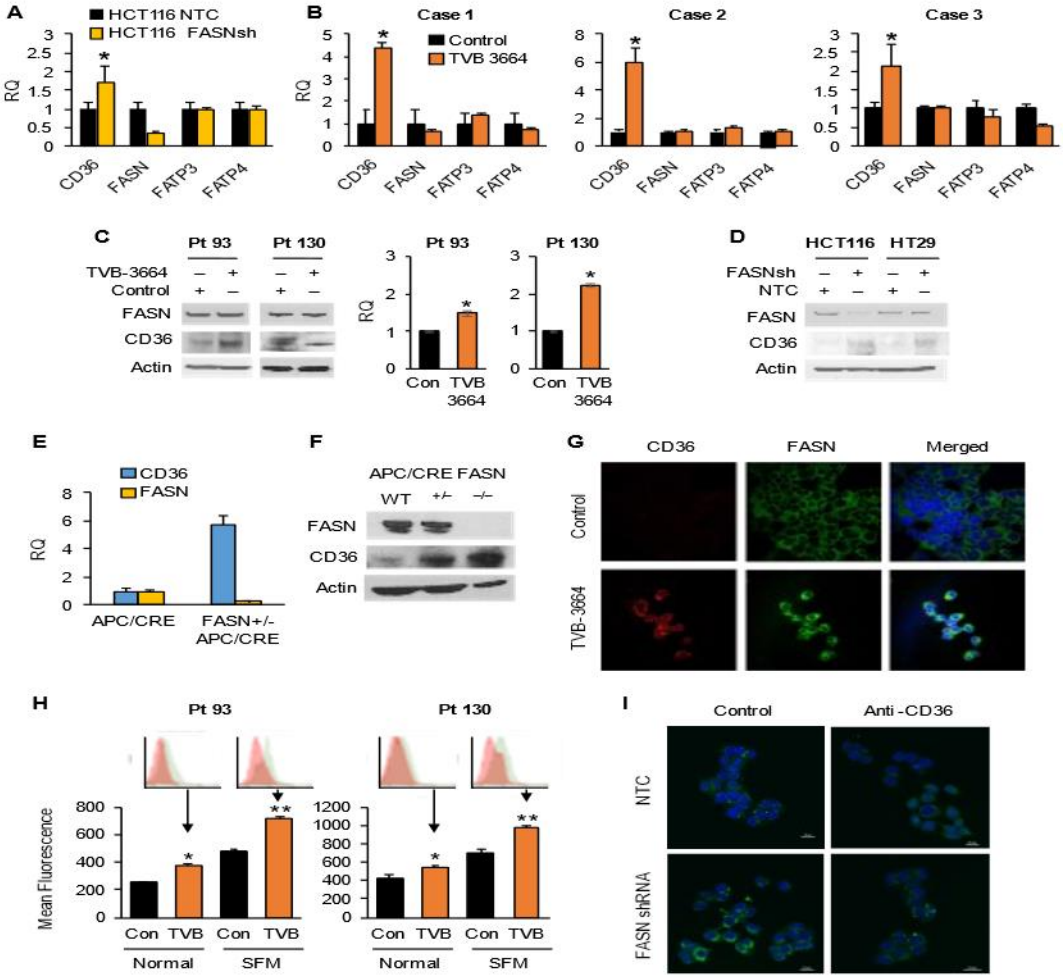


Figure 2.2 Expression of CD36 is selectively regulated by the level of *de novo* FA synthesis in CRC.

(A) shRNA-mediated knockdown of FASN leads to upregulation of CD36 mRNA expression in HCT116 cells. **(B)** TVB-3664 treatment of CRC tissue slices (18h) selectively upregulates CD36 mRNA expression. **(C)** TVB-3664 treatment of Pt 93 and Pt 130 primary CRC cells increases CD36 mRNA and protein expression. **(D)** shRNA mediated knockdown of FASN increases CD36 protein expression in HCT116 and HT29 cells. **(E)** Relative mRNA expression of FASN and CD36 in intestinal tumors collected from APC/Cre and FASN$^{+/-}$/APC/Cre mice. **(F)** FASN and CD36 protein expression in intestinal mucosa collected from Apc/Cre and Apc/Cre mice with hetero- and homozygous deletion of FASN. **(G-H)** Inhibition of FASN increases membrane-associated expression of CD36. **(G)** Confocal images of FASN and CD36 in control and 0.2μM TVB-3664 treated (6 days) Pt 93 primary CRC cells. **(H)** Flow cytometry analysis of Pt 93 and Pt 130 primary CRC cells treated with 0.2uM TVB-3664 (6 days) in normal and serum free media conditions. Mean fluorescence for CD36 is shown for representative data from 3 different experiments (**p<0.01, *p<0.05). (I) FA uptake in HCT116, NTC and FASN shRNA. Cells wre pre-treated with anti-CD36 antibody or vehicle for 24hrs and then treated with BODIPY FL for 10 minutes.

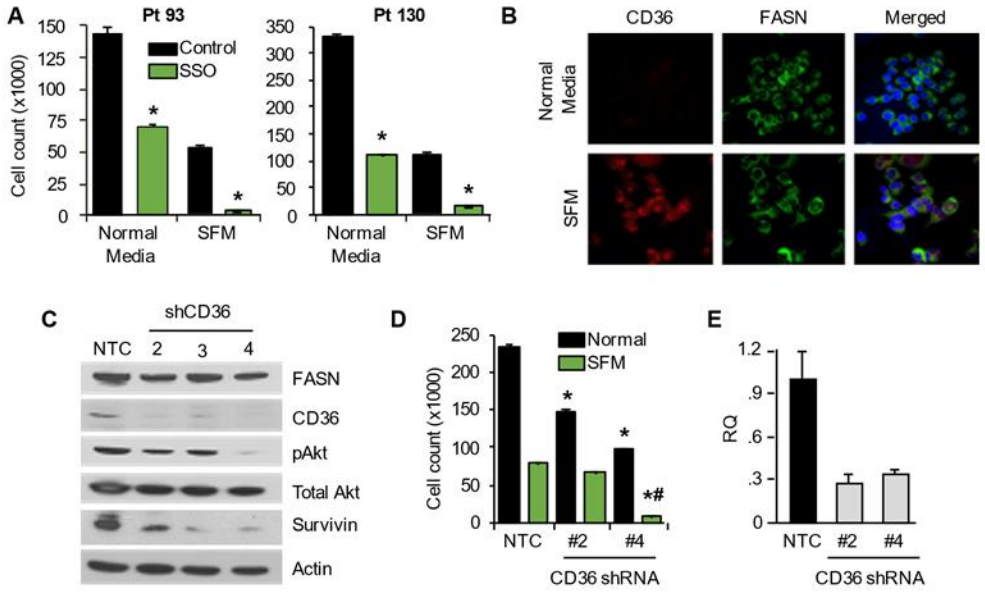


Figure 2.3 Inhibition of CD36 is associated with decreased cellular proliferation.

(A) Primary Pt 93 and Pt 130 CRC cells treated with 100 μM SSO for 6 days. Cellular proliferation assays were performed via cell count. Representative data from 3 experiments is shown (*p<0.05). **(B)** Confocal images of FASN and CD36 in Pt 93 cells in normal and serum free media (6 days). **(C)** Expression of proteins associated with apoptosis and survival in HCT116 transfected with CD36 shRNAs and analyzed via western blot. **D)** Cellular proliferation assay with HCT116, NTC and CD36 shRNA (*p<0.05). (**E)** qRT-PCR confirmation of CD36 knockdown using CD36 shRNA #2 (73%) and CD36 shRNA #4 (67%).

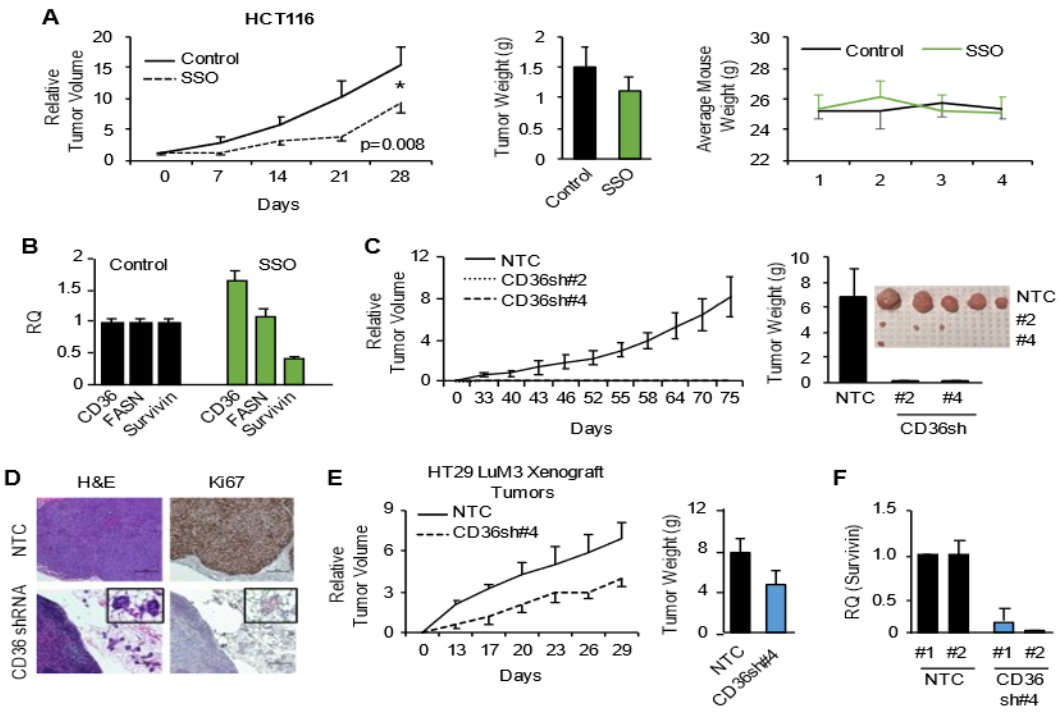


Figure 2.4 SSO treatment and CD36 knockdown inhibit tumor growth *in vivo*.

(A) Tumor volume, tumor weight and mouse weight of control and SSO treated (20mg/kg) mice are shown. SSO was dissolved in 10% PEG and administered in 200 µl dosages via oral gavage daily. 1.0 x 10^6 cells were injected into NU/NU mice. Treatment was initiated when tumors reached approximately 100mm^3 (day 0). **(B)** RT-PCR analysis of HCT116 tumors showing the effect of SSO treatment on CD36, FASN and survivin mRNA expression. (C) Tumor volume of HCT116 NTC and CD36 shRNA #2 and #4 xenografts is shown. 1.0 x 10^6 cells were injected into NU/NU mice and tumor growth was measured every 3 days. **(D)** H&E and Ki67 staining of HCT116 NTC and CD36 shRNA tumors. **(E)** Tumor volume and tumor weight of HT29 LuM3 NTC and CD36 shRNA #4 xenografts are shown. **(F)** mRNA expression of survivin in HT29 LuM3 xenografts (analysis of tumors from 2 mice per group is shown).

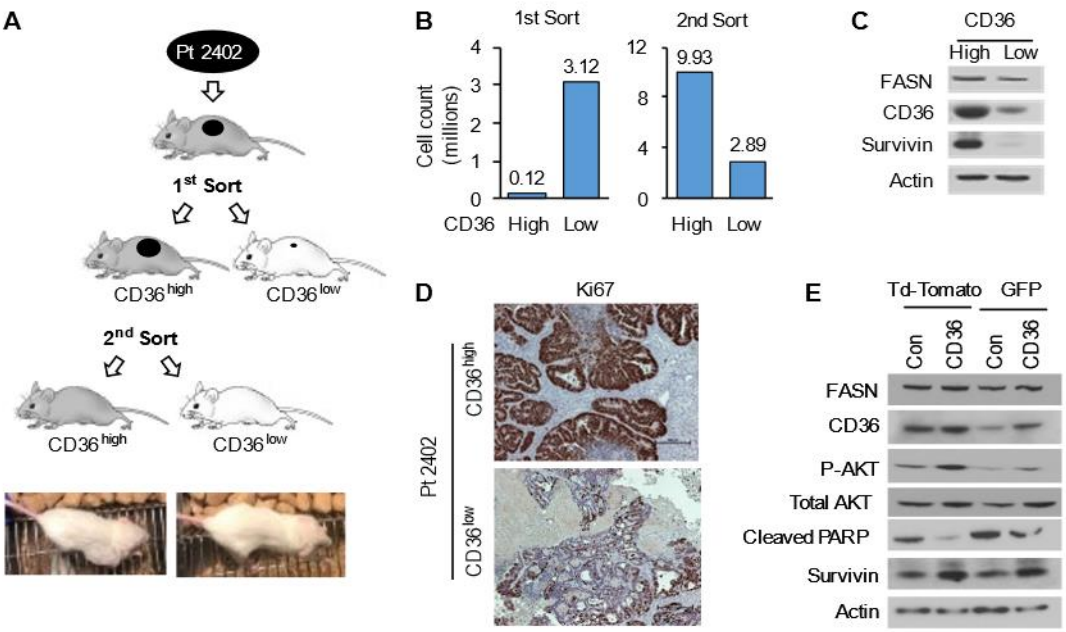


Figure 2.5 High expression of CD36 is associated with an increase in pAkt and survivin in CRC.

(A) Diagram of Pt 2402 propagation after flow cytometry sorting for CD36^{high} and CD36^{low} cells. **(B)** Numbers of CD36^{high} and CD36^{low} Pt 2402 cells for 1st and 2nd flow cytometry sorts. **(C)** Protein expression levels of FASN, CD36 and survivin in CD36^{high} and CD36^{low} Pt 2402 primary cells from 1st flow cytometry sort. **(D)** IHC staining for Ki67 in Pt 2402 CD36^{high} and CD36^{low} tumors. **(E)** Protein expression levels of FASN, CD36, pAkt, cleaved PARP and survivin in HCT116 CRC cells, control and CD36 overexpression.

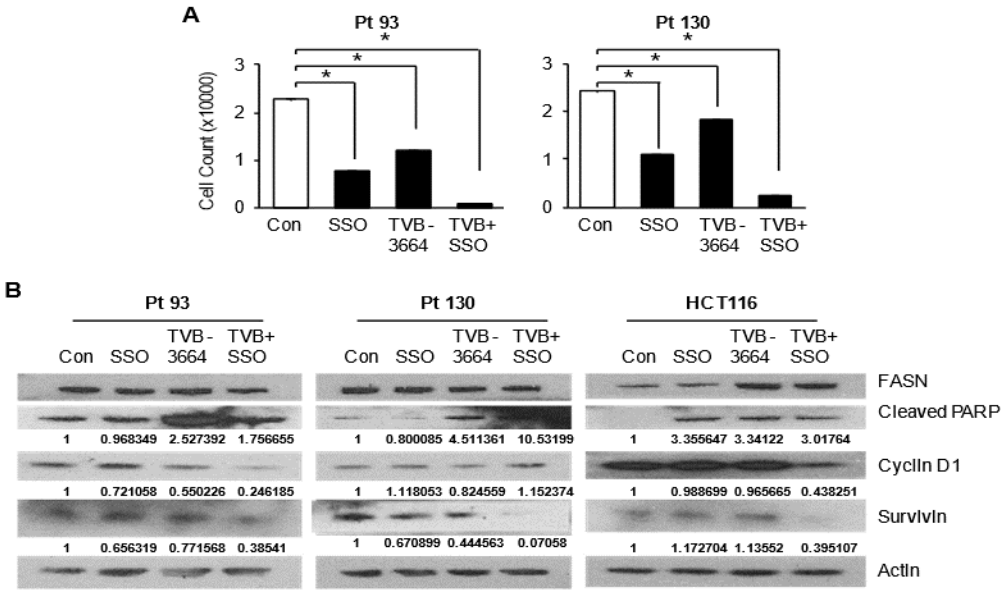
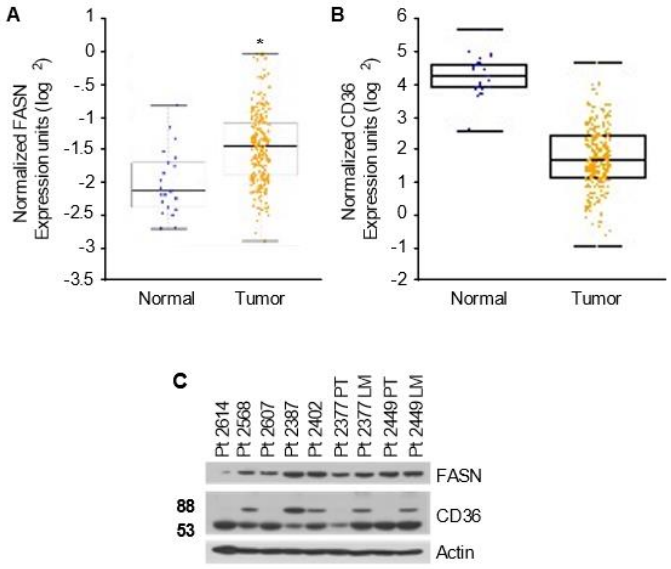
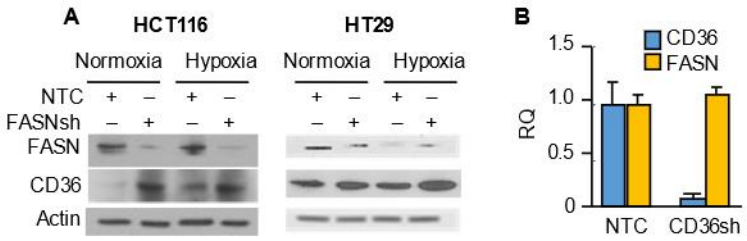


Figure 2.6 Inhibition of CD36 and FASN have a synergetic effect in reducing cell proliferation.

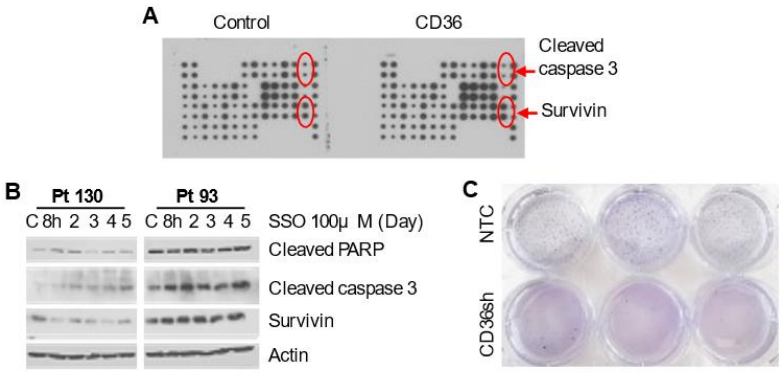
(A) Pt 93, Pt 130 and HCT116 cells were treated with SSO and TVB-3664 alone or in combination for 6 days and cell number was counted. Representative data from 3 experiments is shown (*$p<0.05$). **(B)** Western blot analysis of cells treated with TVB-3664, SSO or TVB-3664 and SSO in combination.



Supplementary Figure S2.1
(A) FASN mRNA expression is increased (*p < 0.0001) and **(B)** CD36 mRNA expression is decreased (*p < 0.0001) in CRC patient samples in the TCGA dataset (n=22 of normal tissues and n=215 of tumors). **(C)** Expression of FASN and CD36 in PDX tumor tissues.

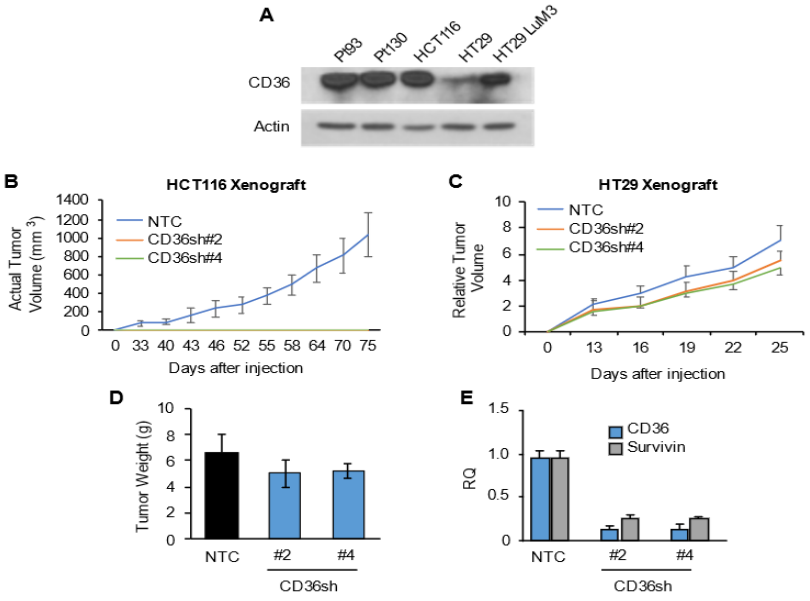


Supplementary Figure S2.2
Expression of CD36 is regulated by the level of *de novo* fatty acid synthesis in CRC. **(A)** Protein expression of CD36 and FASN in FASN shRNA HCT116 and HT29 cells in both normal and hypoxic conditions. **(B)** mRNA expression of CD36 and FASN in HT29LM3 NTC and CD36 shRNA.



Supplementary Figure S2.3

CD36 upregulates survivin and inhibits apoptotic markers. **(A)** Effect of CD36 overexpression on apoptotic markers was assessed in HCT116 cells, control and tdTomato-CD36, using Apoptosis Antibody Array. **(B)** Primary Pt 130 and Pt 93 CRC cells were treated with 100µM SSO for 5 days in normal medium and expression of cleaved PARP, cleaved caspase-3, and survivin was analyzed. **(C)** 3 x 10^4 HT29, NTC and CD36 shRNA #4, cells were plated in agarose and allowed to grow for 10 days.



Supplementary Figure S2.4

(A) Expression of CD36 in CRC cell lines. **(B)** Tumor volume (mm^3) of HCT116, NTC and CD36 shRNA#2 and #4 xenografts shown in Figure 4C. **(C)** Tumor growth for HT29 NTC and CD36 shRNA #2 and #4 xenografts. 2.0 x 10^6 cells were injected into NU/NU mice and tumor growth was measured every 3 days. **(D)** Tumor weight for HT29 NTC and CD36 shRNA #2 and #4 xenografts. **(E)** mRNA expression of CD36 and survivin in HT29 xenograft tumors.

CHAPTER 3. CD36, A FATTY ACID TRANSLOCASE, PROMOTES METASTASIS VIA UPREGULATION OF MMP28 AND AN INCREASE IN E-CADHERIN CLEAVAGE IN COLORECTAL CANCER

3.1 Abstract

Purpose: Altered FA metabolism, a hallmark of cancer, continues to be an attractive target for therapeutic intervention in cancer. Fatty Acid Translocase (CD36) has an important role in FA metabolism as a FA transporter. We have previously shown the importance of CD36 in the promotion of colorectal cancer (CRC) cell proliferation and tumorigenesis as well as its role in the compensation of *de novo* FA synthesis inhibition. CD36 has also been shown to play important roles in migration, invasion and metastasis in gastric, ovarian and oral carcinomas. Matrix metallopeptidase 28 (MMP28) is a member of the metallopeptidase family of proteins which is involved in the degradation of the extracellular matrix in various tissues. MMP28 is relatively novel and poorly investigated in cancer, however it has been shown to be involved in epithelial to mesenchymal transition (EMT) in lung and gastric carcinomas. We have previously shown that cell lines with a higher metastatic potential express higher level of CD36, however, the role of CD36 in CRC metastasis and its potential regulation of EMT has not been previously studied. The purpose of our study is (i) to determine the functional effect of CD36 on CRC metastasis and (ii) to delineate the mechanistic pathways in which CD36 may regulate CRC metastasis.

METHODS: We utilized HCT116 and HT29 CRC cell lines as well as the HT29 LuM3-GFP-Luc cell line, which has been established from the HT29 cell line and has an increased propensity to metastasize to the mouse lung. An increase in metastatic capacity of HT29 LuM3 is associated with an increase in the expression of CD36 and MMP28. We utilize parental HT29-GFP-Luc and HT29 LuM3-GFP-Luc to overexpress and knockdown CD36, respectively. We also use CD36 neutralizing antibody to block activity of CD36 in HCT116 and HT29 LuM3 cells. To measure colonization, migration, and invasion we used soft agar colony formation and trans-well invasion assays. *In vivo* we utilized the tail-vein injection model and the orthotopic cecum injection model. We analyzed samples with qRT-PCR, western blot, confocal microscopy, and immunohistochemistry.

RESULTS: We found that knockdown of CD36 reduces CRC cell invasion and colony formation in HCT116 and HT29 LuM3 *in vitro*. CRC cell invasion and colony formation were increased with the overexpression of CD36 in HCT116 cells. Overexpression of CD36 in CRC cell lines increases mRNA and protein expression of survival and invasion markers including phospho-Akt, as well as MMP28. Knockdown of CD36 in HT29 LuM3-GFP-Luc cells significantly reduced lung metastasis *in vivo* and IHC staining of CD36 knockdown mouse lung tumors show a decrease in CD36 and MMP28 expression. Additionally, CD36 overexpression increased tumor growth and metastasis in cecum injected mice. siRNA mediated knockdown of MMP28 increased e-cadherin expression and decreased the

products of e-cadherin cleavage CTF1 and 2. shRNA knockdown of CD36 decreases expression of MMP28 and significantly increases expression of e-cadherin. Lastly, tissues analyzed from tail-vein injected mice, isogenic PDX tumors with high expression of CD36, and human CRC primary and liver metastasis tissues, show that with an increase in CD36 and MMP28 expression, there is a stark decrease in e-cadherin expression.

CONCLUSION: CRC cells, which have a higher propensity to initiate metastasis *in vivo,* express higher levels of CD36 and MMP28. We show that this upregulation of CD36 and consequently MMP28 represents a unique advantage for CRC cells to initiate tumor colonies, migrate and invade both *in vitro* and *in vivo*. Furthermore, our study highlights the regulation of e-cadherin via cleavage by MMP28 in established CRC cell lines, PDXs, and human CRC tissues, suggesting CD36 via MMP28 may be a desirable therapeutic target for CRC metastasis.

3.2 Introduction

Colorectal cancer (CRC) is the leading cause of non-smoking related cancer deaths and the second leading cause of all cancer related deaths in both the United States and the world [1, 2]. Patients diagnosed with late-stage CRC, Stage III-IV, which exhibit local invasion and distant metastatic disease, have nearly a 10 fold decrease in 5 year survival rate when compared to earlier stage, localized disease [18, 19]. Despite advances in the treatment of primary CRC, there remains a lack of effective therapeutic strategies for late-stage CRC.
Altered FA metabolism is recognized as a hallmark of cancer and multiple studies suggested targeting this pathway as a potential therapeutic strategy for cancer including CRC [128-130]. Fatty acid translocase (CD36) plays a significant role in dietary FA regulation as an exogenous FA transporter [194, 238]. CD36 can be membrane bound, where it can bind low density lipoproteins and extracellular long-chain and ultra-long chain free FAs [194, 272]. CD36 has been implicated in the promotion of primary tumor proliferation and disease progression in multiple malignancies including ovarian, glioblastoma, and breast cancers [149, 151, 241, 273]. Recently, our lab has shown the important role CD36 plays in the proliferation and progression of primary CRC and identified upregulation of CD36 as a potential mechanism of resistance to FASN-targeted therapy [274].

While compelling evidence exists to support CD36's role in the promotion of primary tumor growth and progression in several diseases, there is some evidence to also suggest that CD36 may play a critical role in cancer metastasis. CD36 was shown to promote oral carcinoma migration and invasion *in vitro* and metastasis *in vivo* [150]. In fact, an enhanced presence of tumor cells expressing high levels of CD36 and an increase in metastatic potential is associated with a poor prognosis and clinical outcome in both glioblastoma and oral carcinoma [149, 150]. Additionally, CD36 enhances *in vitro* migration and invasion in gastric cancer cells as well as local invasion and metastasis *in vivo* in ovarian cancer xenografts [151, 242, 273]. Furthermore, CD36 has been shown to promote cell growth and

metastasis as well as epithelial to mesenchymal transition (EMT) in cervical cancer [275, 276]. However, the potential role of CD36 in CRC metastasis has not been previously studied.

EMT is the fundamental process to which an epithelial cell acquires a more mesenchymal phenotype [277, 278]. Aside from the EMT process being utilized during normal tissue development, wound healing, and tissue repair, EMT has also been identified as one of the crucial steps involved in cancer metastasis [279-281]. Various molecular pathways and markers are implicated in the regulation of EMT in CRC, including Snail/Slug, Wnt/B-catenin and particularly the down-regulation of e-cadherin, a critical component of cell-cell adhesion junctions [282].

Loss of e-cadherin is a key characteristic of EMT initiation in various cancers including CRC [223, 283]. E-cadherin expression has previously been shown to be a good prognostic marker for patients with CRC [284]. Furthermore, loss of e-cadherin increases CRC cell invasion and is correlated with poor survival rates of CRC patients [285, 286]. Moreover, lower expression of e-cadherin in CRC patient tumors is associated with tumor differentiation, invasion depth, tumor stage and lymph node metastasis [287]. Although e-cadherin has several know transcriptional regulators including both Snail and Slug, it is suggested that e-cadherin is strictly regulated post-transcriptionally in CRC, either through stabilization or cleavage [288, 289]. However, even with multiple studies showing compelling evidence that lack of e-cadherin expression promotes disease progression, exact mechanisms of e-cadherin loss in CRC are still poorly understood.

The matrix metallopeptidase (MMP) family of proteins have been shown to play an important role in the degradation of several extracellular matrix (ECM) components [290, 291]. MMP28, one of the newest members of this family of proteins to be identified, has been shown to be associated with EMT in lung carcinoma [292]. Furthermore, MMP28 is associated with a poor prognosis and lower survival rates in gastric cancer and increased expression of MMP28 is associated with an increase in invasion and colony formation of gastric cancer cells *in vitro* and *in vivo* [293, 294]. Lastly, MMP28 induced EMT in lung carcinoma is associated with a significant loss of e-cadherin expression [295, 296].

Since previous studies have implicated CD36's involvement in the metastatic process and regulation of EMT in multiple cancers, the goal of this study was to evaluate the contribution of CD36 to CRC metastasis.

We found that CD36 promotes invasion and colony formation of CRC cells *in vitro*. We also found that established HT29LuM3 cell line which have a higher propensity to initiate lung colonies in mice via the tail-vein injection model as compared to parental HT29 cells, express significantly higher levels of CD36 and uptake more FFAs in a CD36 dependent manner [254]. Additionally, we also found that CD36 expression is associated with MMP28 expression in established CRC

cell lines and isogenic patient derived xenografts. Our results also show that knockdown of MMP28 reduces CRC cell invasion *in vitro*. Furthermore, CD36 knockdown significantly reduces lung colony formation *in vivo*. Lastly, we found that CD36 and MMP28 expression are inversely associated with e-cadherin expression in CRC cells. We show that high level of MMP28 expression is associated with an increase in the cleavage of e-cadherin and an increase in the cleavage products of e-cadherin; C-terminus fragment 1 and 2 (CTF1 and CTF2). CTF1 contains both the transmembrane and cytoplasmic domains of e-cadherin and is produced after cleavage [297]. CTF1 can then be processed and cleaved between the cytoplasmic and trans-membrane domains by the Presenilin-1, resulting in the release of CTF2 [297, 298].

Together these findings implicate that CD36 promotes metastasis via upregulation of MMP28 and e-cadherin cleavage and, thus, may be a viable therapeutic target for the treatment of metastatic CRC.

3.3 Results

3.3.1 CD36 Promotes Invasion and Colony Formation in HCT116 Cells

We have previously shown that CD36 promotes CRC proliferation and survival in *in vitro* and *in vivo* [274]. There is also compelling evidence that CD36 contributes to invasion and migration in various cancers including glioblastoma, oral carcinoma, gastric, and ovarian cancer [149-151, 241], but CD36 has not yet been investigated in CRC metastasis. Here, we found that shRNA-mediated knockdown of CD36 in HCT116 cell line leads to a significant reduction in invasion as compared to non-targeted control HCT116 cells (**Figure 3.1 A, D**). Furthermore, when CD36 is overexpressed in HCT116 cells, we observed a significant increase in cell invasion and colony formation (**Figure 3.1 B-D**). Together, these data suggest that CD36 is substantially involved in the invasion and colony formation of established CRC cells.

3.3.2 CD36 Expression is Associated with a More Metastatic Phenotype in Isogenic CRC Cell Lines

To further investigate any potential role CD36 may play in metastasis of CRC, we utilized the HT29 LuM3-GFP-Luciferase trained cell line, a cell line which was serially injected via tail vein in mice and has a significantly higher propensity to initiate lung colonies than the parental HT29 cell line [254]. We have previously shown that CD36 is significantly upregulated in the HT29 LuM3 cell line as compared to parental HT29 [274]. **Figures 3.2 A-B** demonstrate higher CD36 expression HT29 LuM3 cell line compared to parental HT29 using western blot and confocal microscopy, respectively. Additionally, confocal microscopy of FA uptake using BODIPY ™ 558/568 C_{12} demonstrates that HT29 LuM3 cells uptake more free FAs than parental HT29 cells in a CD36-dependent manner **(Figure 3.2 C**). Furthermore, overexpressing CD36 in the parental HT29 cells significantly

increases colony formation and colony diameter (**Figure 3.2 D**). Together these data suggest that CD36 is upregulated in the HT29 LuM3 cell line and associated with acquired more invasive and metastatic behavior of this cell line reported previously [254]. Additionally, the data also suggests that increased expression of CD36 is also associated with an increase in uptake of extracellular FAs.

3.3.3 CD36 Promotes Lung Colonization and Orthotopic Metastasis *in vivo*

To more-thoroughly investigate the role CD36 may play in CRC metastasis, we tested the effect of CD36 on colonization and metastasis *in vivo*. Utilizing the HT29 LuM3 model, we established a HT29 LuM3-GFP-Luciferase non-targeted control (NTC) and CD36 knockdown (shCD36) cell lines and injected these cells via tail vein into NU/NU (n=5 each) mice and monitored for lung colony formation. Mice injected with the HT29 LuM3 shCD36 cell line exhibited significantly lower luciferase reporter bioluminescence signal as compared to mice injected with HT29 LuM3 NTC cells (**Figure 3.3 A**). Furthermore, resected lungs from both NTC and shCD36 injected mice show that mice injected with HT29 shCD36 cells have substantially lower GFP signal and tumor burden than those injected with HT29 NTC (**Figure 3.3 B-C**). The levels of CD36 mRNA expression in these cells are shown in **Figure 3.3 D**.

To better recapitulate the condition of local invasion and metastasis with CRC, we also used the orthotopic cecum injection mouse model. The cecum injection model is well established *in vivo* model which replicates human disease with a higher level of accuracy than other ectopic models [299]. To study CD36's role in CRC metastasis in a more translational way, we injected 1 x 10^6 HCT116 p-Lenti-Control or p-Lenti-CD36 overexpressing cells in 50µl of PBS into the cecum wall of NU/NU (n=5) mice. Our data shows that mice injected with p-Lenti-CD36 overexpressing HCT116 cells exhibited an increase in the number of primary tumors formed compared to mice injected with p-Lenti-Control cells. More importantly, we identified GI metastasis in all mice injected with p-Lenti-CD36 overexpressing cells; however, no metastases were found in mice injected with p-Lenti-Control cells (**Figure 3.3 E-G**). The level of CD36 overexpression in HCT116 cells is shown in **Figure 3.3 H**.

Together, the results of animal experiments support our *in vitro* data and suggest that CD36 significantly contribute to CRC invasion and metastasis.

3.3.4 CD36 is Associated with MMP28 Expression

Gene enrichment analysis of parental HT29 p-Lenti-Control and p-Lenti-CD36 overexpression cell lines show an enrichment of pathways involving focal adhesion, extracellular matrix protein interaction and gap junctions (**Figure 3.4 A**). Additionally, RNA-Seq analysis of both cell lines showed that CD36 expression is associated with matrix metallopeptidase 28 (MMP28) (**Figure 3.4 B**).

MMP28 is the newest member matrix metallopeptidase to be identified and is involved in ECM degradation [292]. MMP28 is associated with metastasis in lung and gastric cancer [295, 296], but has not yet been studied in CRC. To confirm RNA-Seq data, we performed qRT-PCR analysis of HT29 LuM0, p-Lenti-Control and p-Lenti-CD36 overexpression, and HT29 LuM3, NTC and CD36 shRNA, cells. As shown in **Figure 3.4 C**, overexpression of CD36 in HT29 cells leads to significant upregulation of MMP28 mRNA. In contrast, shRNA-mediated knockdown of CD36 in HT29 LuM3 cells leads to a decrease in MMP28 mRNA expression. Consistently, HCT116 cells with CD36 overexpression also exhibit higher levels of MMP28 mRNA expression and inversely, HCT116 CD36 shRNA cells show a significant decrease in MMP28 mRNA expression (**Figure 3.4 D**). Furthermore, HCT116 and HT29 LuM0 cell lines with CD36 overexpression also display higher protein expression of MMP28, and the pro-survival marker p-Akt (**Figure 3.4 E**) [300]. To further support the association between CD36 and MMP28, we show that HT29 LuM3 cells, which express higher levels of CD36, also express higher levels of MMP28 protein compared to the parental HT29 LuM0 cell line as shown by Western blot and confocal microscopy (**Figure 3.4 F-G**).

Together these data show a strong association of CD36 with MMP28 at mRNA and protein levels, suggesting that CD36 may indeed regulate the expression of MMP28.

3.3.5 MMP28 Promotes CRC Cell Invasion and Reduced Expression of E-Cadherin *in vitro*

Previously published data suggests that MMP28 plays a critical role in the regulation of metastasis in lung and gastric cancers. Particularly, MMP28 has been shown to promote EMT in lung carcinoma and increased of expression of MMP28 is associated with a loss of e-cadherin [295, 296]. Loss of e-cadherin is a well-established marker for EMT initiation and poor clinical outcome in various cancers including CRC [283]. MMP28, however, has not been previously studied and it has not been mechanistically investigated in relation to e-cadherin in CRC.

To further elucidate the role of MMP28 in CRC metastasis, we established a transient knockdown of MMP28 in HCT116 cells using MMP28 siRNA. HCT116 siMMP28 cells display a significantly lower ability to invade across a Matrigel Trans-Well as compared to control cells transfected with scrambled siRNA (**Figure 3.5 A**). Furthermore, a decrease in MMP28 mRNA expression due to transfection with MMP28 siRNA leads to a significant increase in mRNA expression of e-cadherin when compared to cells transfected with scrambled siRNA control (**Figure 3.5 B**). Western blot analysis of the HT29-LuM3-GFP-Luciferase NTC and CD36 shRNA knockdown cells demonstrates a reduction in MMP28 expression and an increase in e-cadherin expression further supporting that MMP28 regulates expression of e-cadherin downstream of CD36 (**Figure 3.5 C**). The levels of e-cadherin expression can be regulated transcriptionally or by e-cadherin cleavage and shedding [289]. We did not identify any modes of transcriptional regulation of

e-cadherin, such as through the regulatory proteins Slug or Snail [288, 289], in response to altered expression of CD36 or MMP28. However, to our surprise, when we analyzed our HT29-LuM3-GFP-Luciferase NTC and CD36 shRNA knockdown cells along with control HCT116 cells and cells transfected with MMP28 siRNA by western blot analysis, we found that MMP28 is not only associated with a decrease in functional e-cadherin (135 kD), but also associated with an increase in the cleavage products of e-cadherin, CTF1 and CTF2 **(Figure 3.5 C-D).** While knockdown of CD36 slightly reduced the levels of CTF1, knockdown of MMP28 is associated with almost complete abolishment of e-cadherin cleavage (**Figure 3.5 C-D**) suggesting that MMP28 plays a crucial role in the regulation of e-cadherin, potentially via direct cleavage. These data suggest that CD36 increases the invasive and metastatic potential of CRC cells by regulating EMT via the induction of MMP28 and the cleavage of e-cadherin.

3.3.6 Overexpression of CD36 is Associated with an Increase in MMP28 Expression and a Reduction of E-cadherin *in vivo* and in Human CRC Specimens

To support our data on CD36/MMP28 and e-cadherin axis in CRC metastasis, we analyzed expression of CD36, MMP28 and e-cadherin in lung tissues resected from mice injected with HT29 LuM3, NTC and CD36 shRNA, cells **(Figure 3.3 A).** IHC analysis demonstrates that reduction of CD36 and MMP28 expression is associated with an increase in e-cadherin expression in tumor lesions with CD36 knockdown as compared to NTC tumor tissues **(Figure 3.6 A).**

Our lab has previously shown that isogenic patient derived xenograft (PDX) tumors, which were established from isolating tumor cells expressing high levels of CD36 ($CD36^{high}$), have a higher propensity to grow subcutaneous tumors *in vivo* [274]. To further confirm the relationship between CD36, MMP28 and e-cadherin, we analyzed tissues from Pt2402 $CD36^{high}$ and $CD36^{low}$ PDXs. Western blot analysis and IHC staining confirms that low expression of CD36 is associated with low expression of MMP28 and an increase in the level of e-cadherin **(Figure 3.6 B-C).**

To translate our findings from *in vitro* and *in vivo* to human tissues, we analyzed expression of CD36, MMP28 and e-cadherin in matched normal mucosa, primary CRC and liver metastasis specimens. Our data shows that as we transition from normal tissue to primary tumor and then metastasis, we see an increase in the expression of both CD36 and MMP28 **(Figure 3.6 D)**. Furthermore, we inversely see a decrease in the expression of e-cadherin as we move from normal tissues to primary and metastatic tumors **(Figure 3.6 D)**. In agreeance with our *in vitro* data, these human tissues also show an increase in the cleavage product of e-cadherin, CTF2, as disease progresses, suggesting that MMP28, through CD36 regulation, may directly cleave e-cadherin. Furthermore, when analyzing the expression CD36, MMP28, and e-cadherin in unmatched normal mucosa, primary and metastatic CRC tissues, we see a pattern of expression which is in agreeance with our matched tissue data **(Figure 3.6 E).** Together, this data suggests an

intriguing relationship between CD36 and MMP28, and that of e-cadherin cleavage, further supporting our *in vitro* findings.

3.4 Discussion

Previous investigations from our lab have revealed that CD36 plays an important role in the survival and proliferation of CRC cells and its specific upregulation may be a potential compensation mechanism when *de novo* FA synthesis is inhibited in CRC [274]. Aside from the pro-survival and tumorigenic properties of CD36, CD36 has been implied in the promotion of invasion and metastasis in multiple oncogenic diseases [149-151, 275, 301]. Despite this knowledge, the role of CD36 in the process of invasion and metastasis of CRC has not yet been investigated.

In parallel with previous reports that suggest CRC promotes invasion and metastasis of ovarian and gastric cancer cells *in vitro*, we found that CD36 promotes colony formation and trans-well invasion of CRC cells *in vitro* [151, 242]. We also found that CRC cells which exhibit a more metastatic phenotype express higher levels of CD36 and uptake more exogenous FAs in a CD36-dependent fashion.

One of the premier studies investigating the role of CD36 in metastasis was that of Pascual et al. which described the pro-metastatic characteristic of CD36 in oral carcinoma. It was reported that a subpopulation of oral carcinoma cells which express high levels of CD36 were unique in their ability to initiate metastasis in vivo [150]. Additionally, they found that knockdown or inhibition of CD36 did not affect oral carcinoma primary xenograft tumor growth, but almost completely abolished local invasion [150]. In contrast to their findings, our previous study showed that CD36 does play an important role in primary CRC tumor growth, in particular when endogenously synthesized FAs are limited [274]. However, our current study is in agreeance with their findings on metastasis, suggesting that CD36 is also critical for CRC invasion and metastasis. Consequently, our findings here are also in parallel with several other studies which suggest that CD36 promotes metastasis *in vivo* [149-151, 242].

It is worth noting that due to CD36's role in the survival and proliferation of CRC cells, it is possible that this may also be responsible for the effect of CD36 on CRC metastasis. Therefore, it is critically important to identify a potential mechanistic pathway which distinguishes the pro-survival characteristics of CD36 from any pro-metastatic ones. Previously we described that CD36 is associated with increased levels of the pro-survival marker survivin in primary CRC [274]. We also showed that an increase in expression of CD36 is associated with an increase in phosphorylation of Akt [274]. The Akt pathway is implicated in regulation of both cancer cell survival and metastasis [300]. Previously published study has implicated that CD36 promotes metastatic disease in gastric cancer via the AKT/GSK-3beta/beta-catenin pathway [242]. Consistently with this study, we show

that overexpressing CD36 increases cell invasion and colony formation and is associated with increased levels of p-Akt in established CRC cell lines. However, an increase in phosphorylation of Akt alone is not sufficient to describe a metastatic advantage for CRC.

As previously mentioned, the process of EMT is recognized as one of the crucial steps involved in cancer metastasis and a hallmark of cancer [229, 280, 302]. The remodeling of the extracellular matrix is critical for cells to escape their primary environment and enter a system of circulation (lymph or blood) where they then travel to distant sites and colonize new metastatic tumors [302]. It has been previously shown that increased FA uptake via CD36 is associated with EMT progression in hepatocellular carcinoma cells [222]. Furthermore, CD36 has been shown to promote EMT in cervical cancer through interactions with TGF-beta pathway [220]. More specifically, treatment of cervical cancer cells with TGF-beta increased CD36 expression and exhibited a significant loss in the expression of e-cadherin [220]. Loss of e-cadherin is critical marker for EMT and a well-established hallmark of cancer [302]. Interestingly, our studies show that overexpression of CD36 promotes loss of e-cadherin through a relatively novel member of the MMP family of proteins, MMP28.

A few members of the MMP family of proteins have been linked to EMT in multiple diseases [303-305]. Particularly, MMP-3 and 7 have been shown to promote EMT in breast cancer cells in vitro through direct cleavage of e-cadherin [303, 305]. In most studies, those tumors expressing higher levels of various MMPs result in a poor prognosis for patient survival [304]. As previously mentioned, MMP28 is the newest member of this family of proteins to be identified and is associated with EMT in lung carcinoma [292]. Additionally, MMP28 is associated with invasion and colony formation in gastric cancer cells and increased MMP28 expression is a poor prognostic factor for gastric cancer patients [293, 294]. Our studies show here, for the first time, that CD36 is associated with MMP28 expression in CRC and that MMP28 expression is associated with increased CRC cell invasion and metastasis. We also show here that MMP28 expression is associated with a loss of the critical EMT marker e-cadherin. Along with this loss of e-cadherin, MMP28 expression is associated with increased levels of the cleaved C-terminal fragments of e-cadherin CTF1 and CTF2 [298]. It is worth noting that aside from simply being cleavage products of e-cadherin, the CTFs themselves can act as downstream signaling molecules, such as interacting with and preventing β-catenin degradation as wells as aiding in the translocation of β-catenin to the nucleus, promoting transcription of various downstream genes [306]. Because of the prominent role that the Wnt/ β-catenin pathway plays in CRC, further investigation into the MMP28-e-cadherin-CTF association described here may yield greater information on the mechanisms of CRC progression and metastasis.

Aside from showing just a direct relationship between MMP28 and e-cadherin, we more importantly show that MMP28 downregulation via reduced

expression of CD36 significantly upregulates e-cadherin in CRC cell lines, isogenic PDXs and patient tissues. To our knowledge, this is the first known study to demonstrate the association of CD36 with MMP28 expression and the loss of e-cadherin. Our study is also the first to suggest that MMP28 may be directly involved in the cleavage of e-cadherin in CRC. Therefore, the data presented here show that CD36 may play an important role in the regulation of metastasis in CRC through the upregulation of MMP28 and loss of e-cadherin.

3.5 Conclusions

Late stage CRC, categorized by advanced disease with distant metastasis, remains one of the deadliest cancers in the United States and the world [1]. New therapeutic strategies to identify and target tumor cells with higher metastatic potential is needed to improve the efficacy of treatment and increase overall patient survival. Together, this study highlights the potential of both CD36 and MMP28 as therapeutic targets for CRC. Further investigation into the exact mechanistic regulation of MMP28 by CD36 and MMP28's interaction with e-cadherin is needed to potentially develop new more effective treatment strategies for patients with late-stage CRC or other solid malignancies.

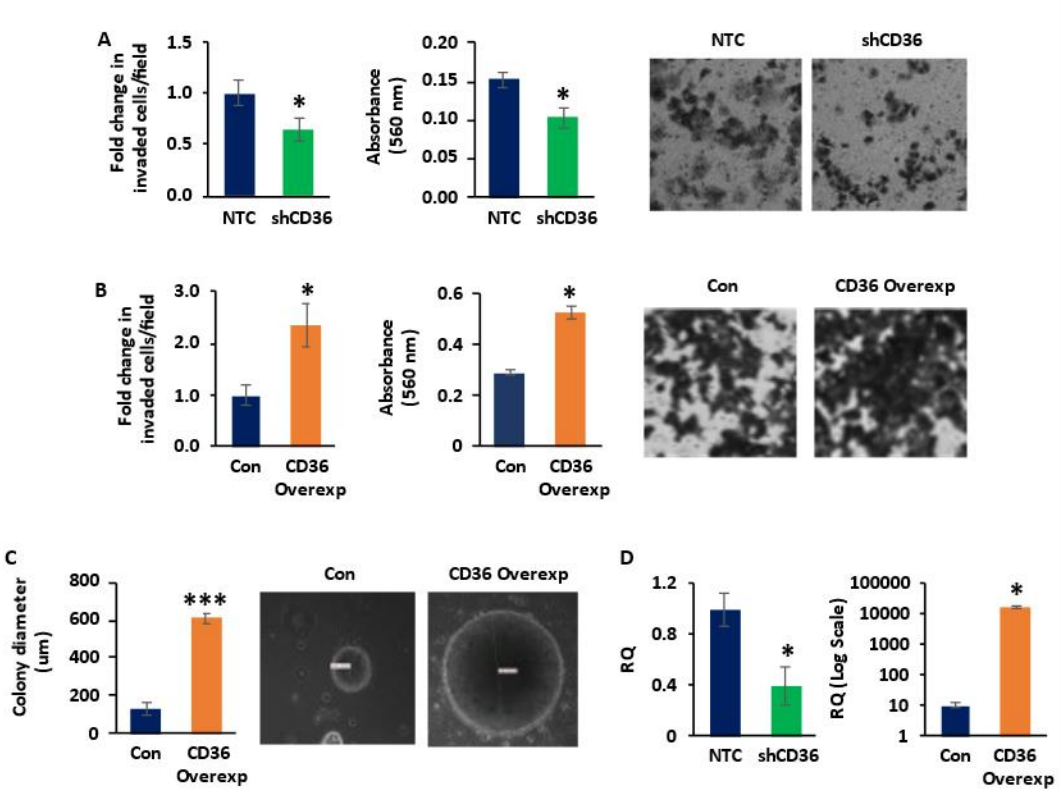


Figure 3.1 CD36 promotes invasion and colony formation in CRC cell lines. **(A)** Matrigel trans-well invasion assay. Average count of invaded cells/field, absorbance at 560 nm of de-stained Matrigel trans-well invasion chambers and raw images of HCT116 NTC and CD36shRNA cell lines are shown. **(B)** Matrigel trans-well invasion assay. Average count of invaded cells/field, absorbance at 560 nm of de-stained Matrigel trans-well invasion chambers and raw images of HCT116 p-Lenti-Control and p-Lenti-CD36 overexpression cell lines are shown. **(C)** Quantification and representative images of HCT116 p-Lenti-Control and p-Lenti-CD36 overexpression soft agar colony formation assays. **(D)** q-RT-PCR demonstrating the levels of knockdown and overexpression CD36 in HCT116 NTC/CD36shRNA and HCT116 p-Lenti-Control and p-Lenti-CD36 overexpression cell lines.

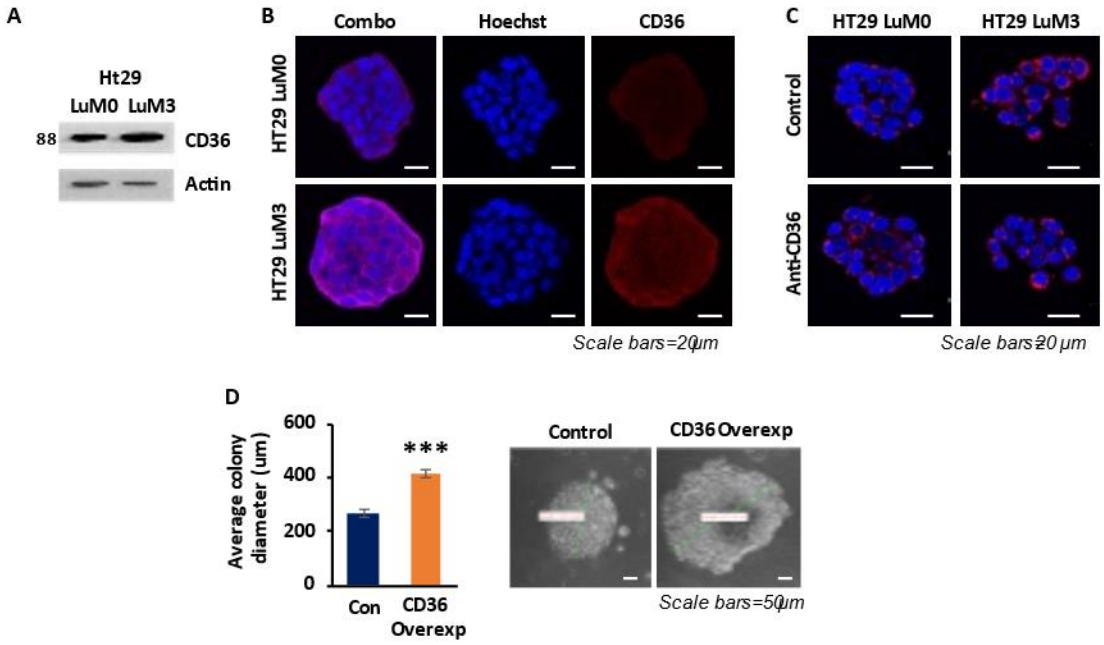


Figure 3.2 CD36 expression is associated with more metastatic CRC cell lines.

(A) Western blot analysis of HT29 LuM0 and LuM3 GFP-Luciferase cell lines. **(B)** Confocal microscopy images of HT29 LuM0 and LuM3 GFP-Luciferase cells for CD36 expression (red), nucleus (blue) and actin filaments (purple). **(C)** FA uptake assay confocal microscopy images of HT29 LuM0 and LuM3 GFP-Luciferase control or treated with anti-CD36 neutralizing antibody for 24h (lipid analogue – red, DAPI – blue). **(D)** Quantification and representative images of HT29 LuM0 p-lenti-control and p-lenti-CD36 overexpression soft agar colony formation assays.

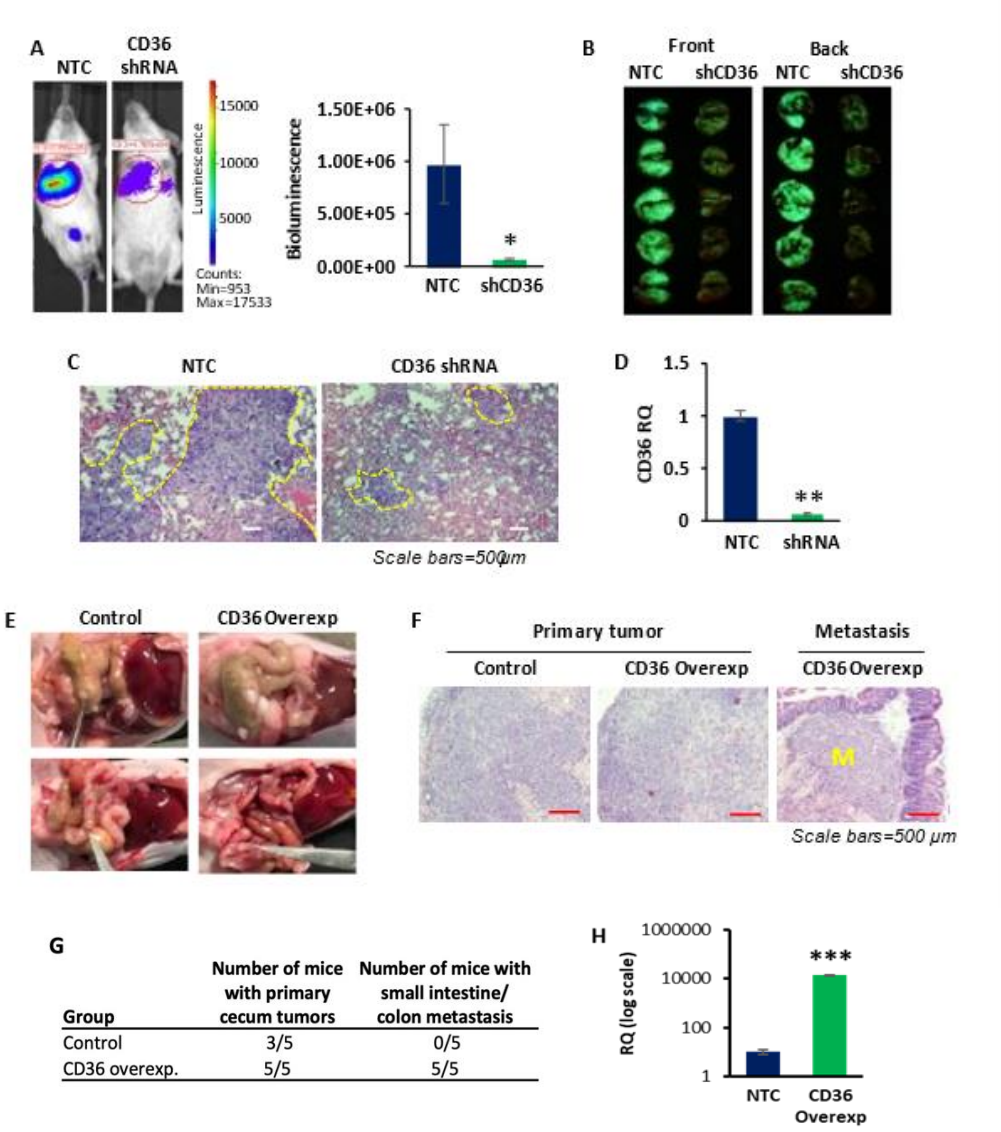


Figure 3.3 CD36 promotes lung colonization and metastasis *in vivo*.
(A) Representative images and quantification of luciferase assay bioluminescence imaging in tail-vein injected mice of HT29 LuM3 GFP-Luciferase cells, NTC (n=5) and CD36shRNA (n=5). **(B)** GFP imaging and **(C)** H&E staining of lung tissues of resected lungs of tail vein injected mice. **(D)** qRT-PCR quantification of CD36 in HT29 LuM3 GFP-Luciferase NTC and CD36sh RNA cell lines. **(E)** Images from HCT116 control and CD36 overexpression cecum injected mice showing increased tumor burden and colon metastasis in the CD36 overexpression mice. **(F)** H&E staining of the resected primary and metastatic tissues from mice injected with control and CD36 overexpression HCT116 cells (M–metastasis). **(G)** Quantitative analysis of primary cecum tumors and GI metastasis. **(H)** qRT-PCR quantification of CD36 in HCT116 p-Lenti-Control and CD36-Overexpression cell lines. (*p <.05, ** p <.01, ***p <.001)

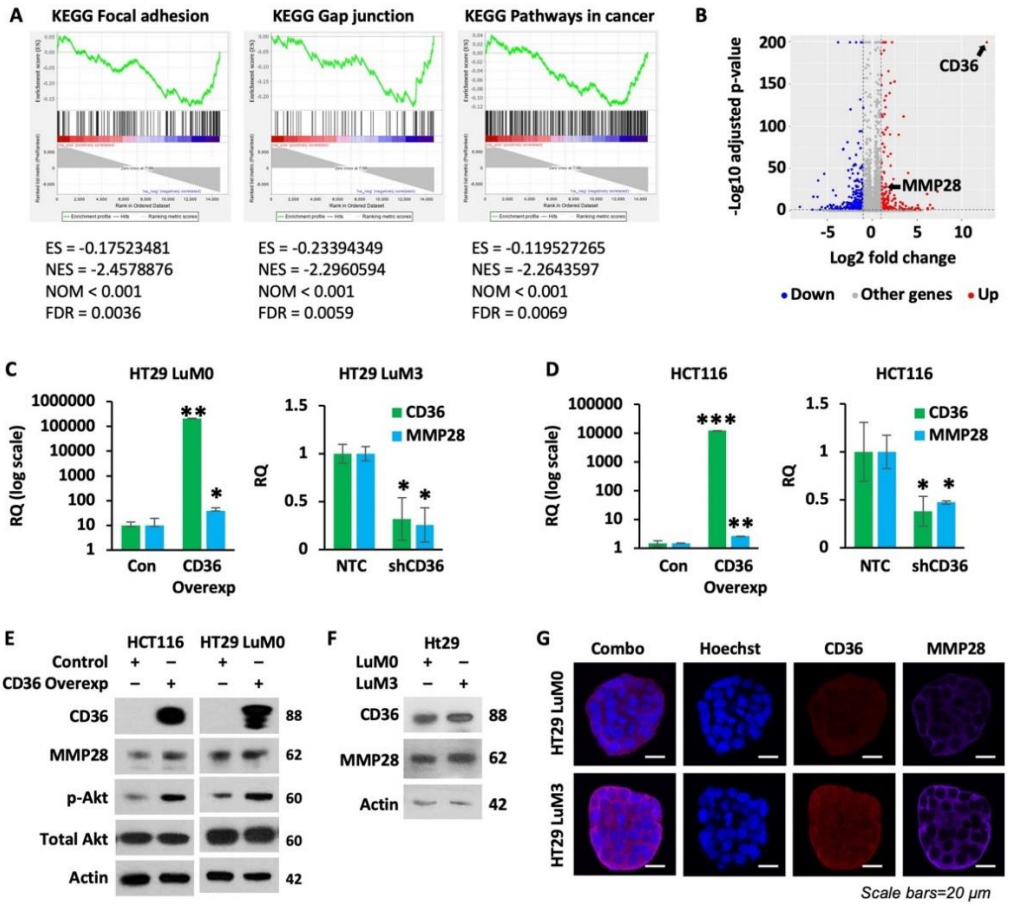


Figure 3.4 CD36 regulates expression of MMP28.

(A) Representative gene set enrichment analysis (GSEA) plots generated from RNA-seq expression data of HT29 p-Lenti Control and HT29 p-Lenti CD36 overexpression cell lines. The top 10 enriched pathway sets are provided in **Supplemental Figure 3.2**. Bar codes indicate the location of gene set members in the ranked list of all genes. ES, enrichment score; NES, normalized enrichment score; NOM, nominal p-value; FDR, false discovery adjusted p-value. **(B)** Volcano plot of HT29 p-Lenti Control and HT29 p-Lenti CD36 overexpression cell lines showing increased levels of CD36 mRNA associated with an increase in MMP28 mRNA expression. **(C)** qRT-PCR analysis of CD36 and MMP28 mRNA in HT29, p-Lenti Control and p-Lenti CD36 overexpression cell lines, and in HT29 LuM3 GFP-Luciferase NTC and shCD36 cell lines. **(D)** qRT-PCR analysis of CD36 and MMP28 mRNA in HCT116, p-Lenti Control and p-Lenti CD36 overexpression, and HCT116, NTC and CD36 shRNA cell lines. **(E)** Western blot analysis of HCT116 and HT29 LuM0 p-Lenti Control and p-Lenti CD36 overexpression cell lines for CD36, MMP28, p-Akt and total Akt. **(F)** Western blot of analysis of CD36 and MMP28 in HT29 LuM0 and HT29 LuM3 GFP-Luciferase cell lines. **(G)** Confocal microscopy of HT29 LuM0 and HT29 LuM3 GFP-Luciferase cell lines for actin, CD36 and MMP28 (*p <.05, ** p <.01, ***p <.001).

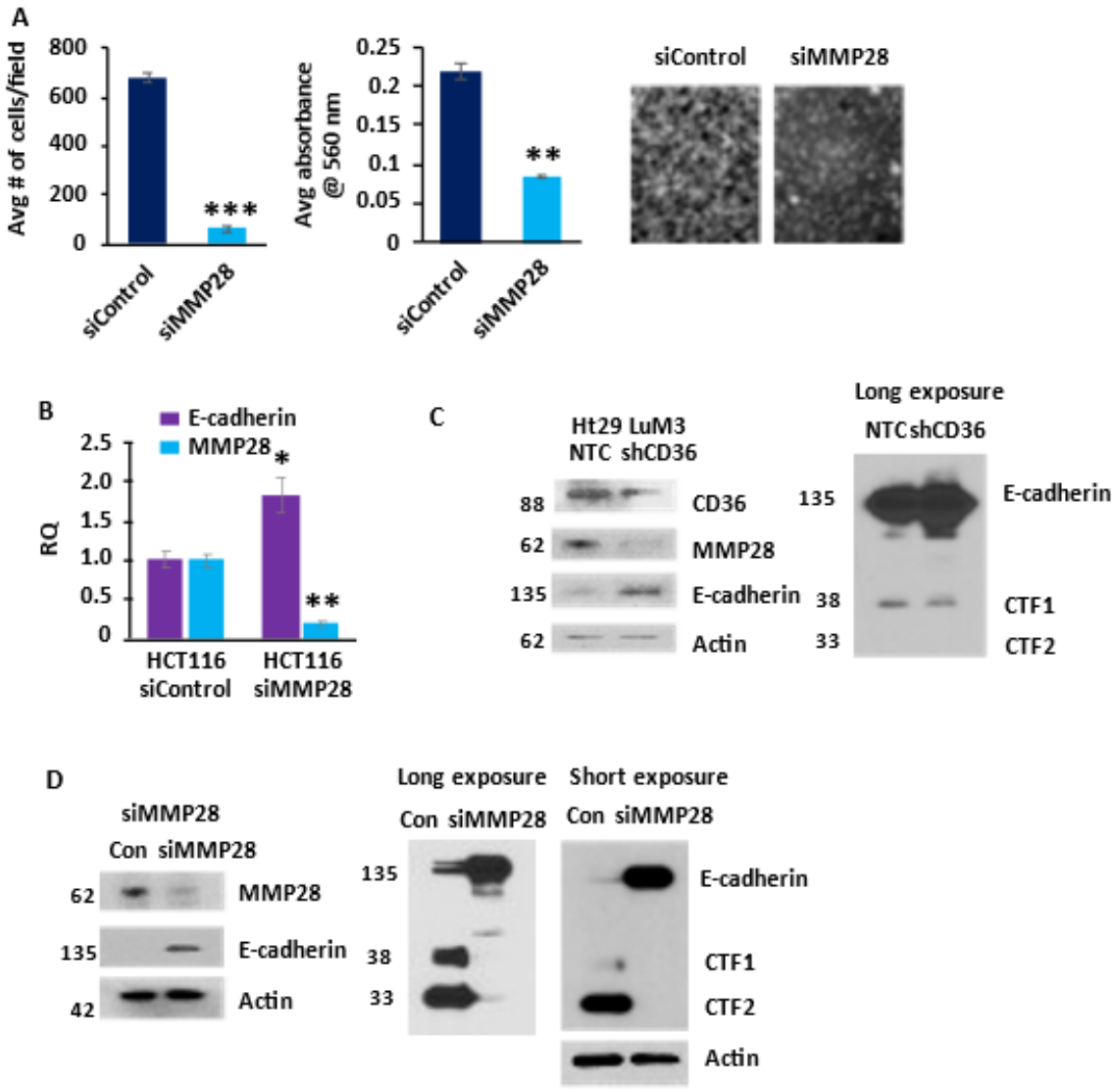


Figure 3.5 MMP28 reduces CRC cell invasion and decreases expression of functional e-cadherin *in vitro.*

(A) Normalized average count of invaded cells/field, absorbance at 560 nm of de-stained Matrigel trans-well invasion chambers and raw images of Matrigel trans-well invasion chambers of HCT116, siControl and siMMP28 cell lines. **(B)** qRT-PCR analysis of control and MMP28 siRNA transfected HCT116 cell lines for MMP28 and e-cadherin. **(C)** Western blot analysis of HT29 LuM3 NTC and shCD36 cell lines for CD36, MMP28 and e-cadherin. **(D)** Western blot for MMP28 and e-cadherin expression. Western blots including e-cadherin cleavage products CTF1 and CTF2 are shown on separate blots (long and short exposure). (*p <.05, ** p <.01).

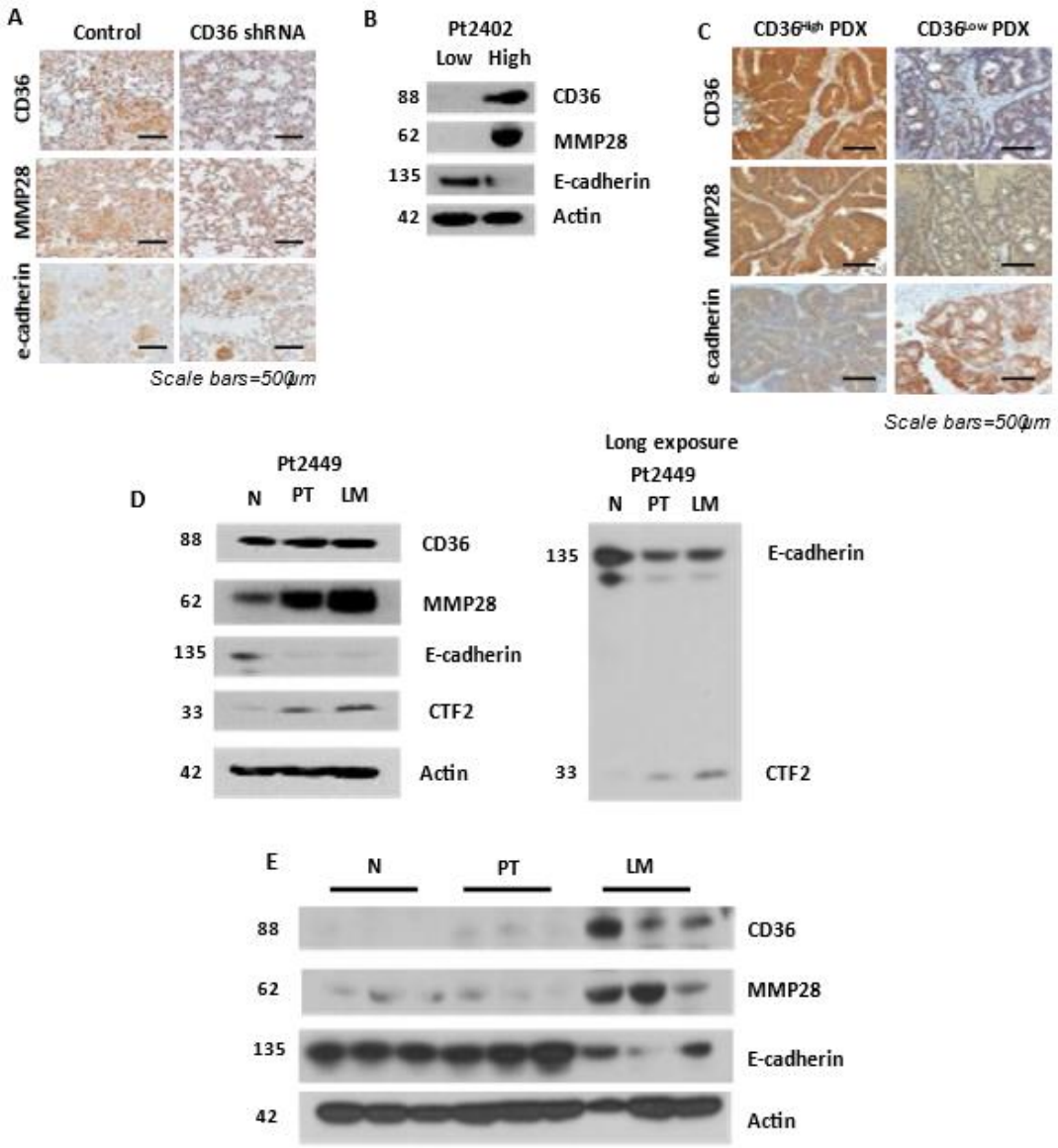


Figure 3.6 Overexpression of CD36 is associated with an increase in MMP28 expression and reduction in the level of e-cadherin in vivo and human CRC specimens.

(A) IHC analysis of tissues from tail vein injection of HT29 LuM3 GFP-Luciferase NTC and shCD36 cell lines shown in Figure 3A. **(B)** Western blot analysis and **(C)** IHC analysis of tissues from Pt2402 PDX, CD36 low and CD36 high, isogenic tumors for CD36, MM28, and e-cadherin. **(D)** Western blot analysis of matched normal (N), primary tumor (PT) and liver metastasis (LM) tissues for CD36, MMP28, e-cadherin, and CTF2. **(E)** Western blot analysis of CD36, MMP28, and e-cadherin in unmatched N, PT, and LM patient tissues.

CHAPTER 4. FATTY ACID SYNTHASE PROMOTES TUMOR GROWTH VIA UPREGULATION OF HEXOSAMINE METABOLISM AND O-LINKED GLYCOSYLATION IN COLORECTAL CANCER

4.1 Abstract

PURPOSE: The Adenomatous polyposis coli (APC) gene is an important regulatory gene involved in Wnt/β-catenin signaling. Mutations in the APC gene are associated with CRC tumorigenesis and progression. Animal models have been developed to study APC-driven tumorigenesis *in vivo*. Our lab has previously demonstrated the role of FASN in CRC proliferation and tumorigenesis. However, FASN has not yet been investigated in regard to APC-driven tumorigenesis. Therefore, the purpose of this study was to: (i) determine the effect of FASN deletion on adenoma formation and mice survival in the APC/Villin-Cre mouse model and (ii) deliniate potential signaling and metabolic pathways effected by altered FASN expression which may promote tumorigenesis in the APC/Villin-Cre mouse model.

METHODS: We have developed mouse colonies bred from the APC/Villin-Cre mouse model in combination with mice containing floxP FASN, resulting in FASN/Apc/Villin-Cre, $FASN^{+/\Delta}$/Apc/Villin-Cre, and $FASN^{\Delta/\Delta}$/Apc/Villin-Cre strains. Mice from FASN/Apc/Villin-Cre and $FASN^{+/\Delta}$/Apc/Villin-Cre strains, both male and female, were monitored for survival duration. Intestinal tissues from each strain were also analyzed for FASN expression via IHC and qRT-PCR and adenomas were counted on the sections of small intestine. RNA transcriptomes from adenomas collected from all three mouse strains were analyzed via RNA-seq analysis. Utilizing the list of identified differentially expressed genes (DEGs), the Gene enrichment analysis was performed. Metabolomic analysis of adenomas collected from FASN/Apc/Villin-Cre, $FASN^{+/\Delta}$/Apc/Villin-Cre, and $FASN^{\Delta/\Delta}$/Apc/Villin-Cre mice was performed using gas chromatography-mass spectrometry. The protein expression of metabolic enzymes in these mice was analyzed using the Reverse Phase Protein Array (RPPA). The primary cell lines, Pt 93 and P t130, were treated with GFPT1 inhibitor azaserine and OGT inhibitor OSMI1, and cell proliferation and colony formation assays performed. The established CRC cell line HCT116 was used to knockdown GFPT1 and OGT and utilized for subcutaneous xenografts *in vivo.*

RESULTS: The high level of FASN expression is seen intestinal adenomas developed in Apc/Villin-Cre mice. Heterozygous deletion of FASN leads to a significant increase in survival of both male and female mice. Consistently, significantly fewer adenomas formed in Apc/Cre mice with heterozygous deletion of FASN as compared to APC/Villin-Cre control mice. RNA-seq and gene enrichment analysis of tissues from FASN/Apc/Villin-Cre, $FASN^{+/\Delta}$/Apc/Villin-Cre, and $FASN^{\Delta/\Delta}$/Apc/Villin-Cre mice show a drastic change in global gene expression including alterations in pathways involved in cell proliferation, energy metabolism and CRC initiation and progression. Metabolic analysis of these tissues also

reveals significant decrease in abundance of multiple metabolites involved in the TCA cycle, glycolysis, and hexosamine biosynthesis. Importantly, the RPPA data demonstrate the deletion of FASN in mouse is associated with a significant decrease in expression of glutamine-fructose-6-phosphate aminotransferase (GFPT1) and O-Linked N-Acetylglucosamine Transferase (OGT), enzymes within the hexosamine biosynthesis pathway. More specifically, knockout of FASN reduces the mRNA and protein expression of both GFPT1 and OGT. Furthermore, inhibition of GFPT1 and OGT reduces primary CRC cell proliferation and colony formation *in vitro*. Lastly, knockdown of GFPT1 and OGT in HCT116 cells reduces tumor growth *in vivo*.

CONCLUSIONS: Our data here shows that FASN promotes tumorigenesis in APC/Cre mouse model. Additionally, FASN also regulates multiple metabolic processes in these mouse strains, including that of hexosamine biosynthesis. Furthermore, we found that an increase in GFPT1 and OGT expression promotes CRC cell proliferation, colony formation and tumor growth. Together, this data further supports the fact that FASN is a desirable therapeutic target for CRC. Interestingly, our data here suggests that targeting the regulation of metabolic processes other than FA metabolism, such as hexosamine biosynthesis, by FASN, could potentially lead to the development of more efficacious treatment strategies in CRC. Lastly, our study emphasizes that further investigation into hexosamine biosynthesis and its regulation is needed to fully delineate the role this pathway plays in CRC progression.

4.2 Introduction

Colorectal cancer (CRC) is currently the third most diagnosed cancer and the second most common cause of cancer related deaths in the world [1, 307]. CRC is primarily sporadic, resulting from a variety of environmental factors such as smoking and obesity, but can also be linked to genetic inheritance [308]. The Adenomatous polyposis coli (APC) gene is an important regulatory gene which binds to and sequesters β-catenin in the cytoplasm of the cell, preventing β-catenin translocation to the nucleus where it would then promote translocation of various pro-survival and proliferation genes [309]. Mutations in APC have been shown to be necessary for initiation of familial adenomatous polyposis (FAP) and mutated APC is suggested in the role of a driver mutation for CRC [309, 310].

Several *in vivo* models have been developed in an attempt to more accurately investigate CRC initiation, progression, and metastasis. Some such models include mutations in and/or alteration in the expression of the APC gene [310]. A prominent mouse model focusing on APC is the APC multiple intestinal neoplasia ($Apc^{Min/+}$) mouse model, in which the APC gene has a truncation mutation in codon 850. Mice with heterozygous min mutation in APC develop multiple small intestinal polyps which can progress to adenocarcinoma [311]. This model is an excellent experimental representation of APC driven FAP and sporadic colon adenoma development in humans [312].

Another prominent *in vivo* model targeting APC is the APC/Villin-Cre mouse model, in which the APC gene is flanked by loxP sequences and Cre recombinase is expressed within the crypt epithelial cells of the small and large intestine using the intestinal specific promoter sequence of Villin [313]. This results in intestinal specific deletion of APC, which thus leads to a phenotype comparable to that of the Apc^{Min} mouse model [311, 313]. With this model, it is possible to study APC-driven tumorigenesis *in vivo*, and potentially identify other pathways which contribute to this phenomenon.

Altered metabolism is commonly exhibited in CRC and is a hallmark of cancer and a potential target for therapeutic intervention [128-130, 314, 315]. Fatty acid synthase (FASN), a critically important enzyme of *de novo* lipogenesis, has previously been shown to be upregulated in CRC and involved in tumor progression and metastasis.[153, 231] Previous studies from our lab show significant reduction of tumor growth in patient derived xenografts (PDXs) and primary cell proliferation *in vitro* when treated with TVB, a novel inhibitor of FASN developed by Sagimet Biosciences and currently in phase I/II clinical trials [185, 191-193]. However, the role FASN may play in CRC tumor initiation and development has not yet been studied.

Hexosamine metabolism involves the process known as glycosylation, in which a glycan is attached to a protein or other functional molecular target and is an important metabolic pathway involved in the development and regulation of various tissues and cellular processes [316]. This complex metabolic pathway is a branch of glycolysis and involves several enzymes. These include the entry and rate limiting enzyme glutamine-fructose-6-phosphate aminotransferase (GFPT1) as well as O-Linked N-Acetylglucosamine Transferase (OGT), which attaches the glycan (β-D-N-acetlyglucosamine (GlcNAc), to a hydroxyl group of serine or threonine moieties of molecular targets (O-GlcNAc) [316-318]. The resulting proteins now containing an O-GlcNAc moiety are then used in a variety of cellular functions including cell signaling and metabolic flux. [317, 319, 320]. Altered levels of O-GLcNAc have been shown to correlate with cancer progression and altered glycosylation is also a hallmark of cancer [321, 322]. Furthermore, aberrant protein glycosylation is a pathological alteration that is frequently associated with onset and progression of CRC, cancer cells immunogenicity and drug resistance [323-325]. It is suggested that O-GlcNAc may be directly modulated by several processes including FA metabolism [326]. However, any role of *de novo* lipid synthesis via FASN in the regulation of cellular glycosylation and their combined role in CRC has not been previously studied.

We have bred an APC/Villin-CRE mouse along with mice containing a loxP-flanked FASN gene expressed with the Villin promoter (FASN/Villin-Cre) resulting in a colony of mice in which both APC can be genetically deleted along with heterozygous or homozygous deletion of FASN ($FASN^{+/\Delta}$ /Apc/Villin-Cre and $FASN^{\Delta/\Delta}$ /Apc/Villin-Cre). We found that mice containing heterozygous deletion of

FASN in combination with APC deletion live significantly longer than those with APC deletion alone. Furthermore, we show that both heterozygous and homozygous deletion of FASN in APC/Villin-Cre mice result in significantly lower numbers of adenomas in the intestine compared to APC/Cre control. Consistantly, we demonstrate that deletion of FASN downregulates several pathways including those involved in cell proliferation, energy metabolism, and CRC initiation and progression. More specifically, metabolic analysis of mouse tissues with FASN heterozygous and homozygous deletion reveals a significant alteration of multiple metabolites involved in glycolysis and the citric acid cycle (TCA). We further show that FASN regulates important enzymes in hexosamine biosynthesis, specifically GFPT1 and OGT. Deletion of FASN in mouse tissues significantly downregulates both OGT and GFPT1 as well as the product of glycosylation, O-GlcNAc. Lastly, we demonstrate that inhibition of glycosylation through the targeting of OGT and GFPT1 inhibits CRC cell proliferation, colony formation, and xenograft tumor growth *in vivo.*

Together, the data presented here shows that FASN plays a significant role in the initiation of adenomas in the APC/Villin-Cre mouse model. Additionally, these results suggest that FASN may regulate hexosamine biosynthesis, which is an important metabolic pathway in the proliferation and progression of CRC. These results emphasize the need for further investigation into the mechanistic regulation of hexosamine biosynthesis by FASN. Furthermore, these results may provide further insight into the development of new, more-effective therapeutics against CRC through the targeting of FASN, hexosamine biosynthesis, or both.

4.3 Results

4.3.1 Heterozygous Deletion of FASN Increases Survival and Decreases Adenoma Burden *in vivo*

To analyze the potential role of FASN on CRC initiation and adenoma formation, we utilize the APC/Villin-Cre and FASN/Apc/Villin-Cre mouse models. IHC analysis of tissues from APC/Villin-Cre mice show that FASN is highly expressed in tumor tissues **(Figure 4.1 A).** C57BL/6J mice with LoxP-flanked FASN alleles were bred with APC/Villin-Cre mice to produce FASN/Villin-Cre, $FASN^{+/\Delta}$/Apc/Villin-Cre, and $FASN^{\Delta/\Delta}$/Apc/Villin-Cre mouse colonies which exhibit a significant reduction in FASN mRNA expression **(Figure 4.1 B).** We show that both male and female mice with heterozygous deletion of FASN live significantly longer than their APC/Villin-Cre counterparts **(Figure 4.1 C).** Additionally, $FASN^{+/\Delta}$/Apc/Villin-Cre mice, both males and females, exhibit significant lower adenoma burden compared to APC/Villin-Cre mice alone **(Figure 4.1 D-E).**

Together, these data suggest that FASN is an important mediator of tumor initiation in the APC/Villin-Cre mouse model, implying that CD36 may play an important role in the initiation of CRC in human patients.

4.3.2 FASN Deletion Significantly Alters Cellular Gene Expression Profile; Decreasing Expression of Genes Enriched in Protein Synthesis, Cell Cycle, and Energy-generating Metabolic Pathways

To delineate the mechanism behind the phenotype observed in the FASN/Apc/ Villin-Cre mouse model, we performed RNA-seq analysis from tissues taken from the mouse lines described above. RNA-seq analysis revealed significant changes in mRNA expression of numerous genes between the APC/Villin-Cre, $FASN^{+/\Delta}$ /Apc/Villin-Cre, and $FASN^{\Delta/\Delta}$ /Apc/Villin-Cre mouse tissues **(Figure 4.2 A-B).** Because of this, we also performed gene enrichment analysis of these mouse tissues which demonstrated significant changes in the expression of genes involves in cell proliferation, energy metabolism and CRC initiation and progression **(Figure 4.2 C),** thus, supporting our data in **Figure 4.1.**

4.3.3 FASN Knockout Alters Cellular Metabolism

Further analysis of tissues collected from the mouse lines described above showed several alterations in genes involved in various cellular metabolic processes. Reverse phase protein array (RPPA) analysis revealed significant changes in genes involved in the TCA cycle, glycolysis, and hexosamine biosynthesis **(Figure 4.3 A-B).** Analysis of cellular metabolites was performed using gas chromatography-mass spectrometry (GCMS). **(Figure 4.3 C).** We are currently analyzing this data and have already identified significant changes in metabolites within glycolysis and its branching pathways and TCA cycle.

Together, these data suggest that FASN is an important regulator of not only FA metabolism directly, but also various other metabolic processes in CRC, which could have significant impact on CRC cell growth.

4.3.4 Overexpression of FASN Increases Expression of GFPT1 and OGT *in vivo*

Altered glycosylation is frequently associated with the onset and progression of CRC [323-325]. Interestingly, RPPA analysis showed that hexosamine biosynthesis was altered in FASN knockout mouse tissues, specifically two critical genes necessary for hexosamine biosynthesis, GFPT1 and OGT **(Figure 4.4 A)**. To further elaborate on this, we analyzed protein expression of genes related to hexosamine biosynthesis as well as the product of this pathway, O-GlcNAc, and found that several genes and O-GlcNAc were downregulated in FASN knockout mouse tissues **(Figure 4.4 B-C)**.

Our lab has also established an inducible CRE mouse model, where the Cre recombinase enzyme is expressed with an ERT2 promoter, allowing for expression of Cre only in the presence of tamoxifen. When bred with our FASN mouse strains, the results are the, $FASN^{+/\Delta}$ /Apc/Villin-Cre -ERT2 and $FASN^{\Delta/\Delta}$ /Apc/Villin-Cre-ERT2 strains, which upon administration of tamoxifen, both FASN and APC are deleted from the genome. Utilizing this model, we analyzed the

mRNA and protein expression levels of GFPT1 and OGT and found that when FASN is knocked out via tamoxifen, both GFPT1 and OGT expression is significantly reduced and comparable to that of the $FASN^{+/\Delta}$ /Apc/Villin-Cre and $FASN^{\Delta/\Delta}$ /Apc/Villin-Cre strains **(Figure 4.4 D-E)**.

These data suggest that FASN significantly regulates hexosamine biosynthesis via the regulation of GFPT1 and OGT gene expression, implying that FASN may regulate CRC initiation and progression not only through direct FA metabolism, but also through glycolysis.

4.3.5 High Expression of GFPT1 and OGT is associated with an increase in CRC Proliferation and Tumorigenesis

To delineate the effect of hexosamine biosynthesis in CRC, we utilized the primary CRC cell line Pt93 established in our lab. The drug Azaserine is a chemical inhibitor of GFPT1 [327]. Treatment of Pt93 with varying concentrations of Azaserine results in a significant reduction in cell proliferation **(Figure 4.5 A)**. Additionally, treatment of Pt93 cells with OSMI-1, a chemical inhibitor of OGT, also significantly reduced proliferation **(Figure 4.5 A)**. Furthermore, treatment of both drugs on Pt93 also greatly reduced soft agar colony formation **(Figure 4.5 B)**. Moreover, knockdown of GFPT1 and OGT in the established CRC cell line HCT116 reduced subcutaneous xenograft tumor volume and tumor weight *in vivo* **(Figure 4.5 C-D)**.

Cumulatively, this data suggests that hexosamine biosynthesis is a critical component to CRC proliferation and progression and that the enzymes GFPT1 and OGT play a crucial role in this process.

4.4 Discussion

The APC/Villin-Cre mouse model allows investigators to study APC driven adenoma formation in vivo [312]. Previous studies from our lab have demonstrated the role of FASN in CRC cell proliferation and tumor growth. [153, 185]. However, FASN has never been investigated regarding APC driven adenoma formation in animal models. Here for the first time, we demonstrate that FASN is significantly upregulated in APC/Villin-CRE mice. Additionally, we established $FASN^{+/\Delta}$ /Apc/Villin-Cre and $FASN^{\Delta/\Delta}$ /Apc/Villin-Cre mouse strains and found that when FASN expression is reduced, these mice exhibit significantly reduced tumor burden and survive longer than their APC/Villin-CRE counterparts. Additionally, through RNA-seq, gene enrichment, and RPPA analysis, we show that FASN knockout reduces the expression of several metabolic genes involved in the TCA cycle, glycolysis, and hexosamine biosynthesis.

Dysregulation of hexosamine biosynthesis, otherwise known as glycosylation, is frequently associated with the onset and progression of CRC [323-325]. Two prominent enzymes involved in glycosylation are GFPT1, the rate

limiting enzyme, and OGT, the enzyme responsible for the final step of glycosylation, producing O-GlcNAc [316-318]. It is suggested that levels of cellular O-GlcNAc may be directly regulated by FA metabolism. However, the role of GFPT1 and OGT in disease progression as well as any potential regulation of glycosylation by FASN has not yet been investigated in CRC. Our data here shows that when FASN expression is reduced is mouse tissues, both GFPT1 and OGT mRNA and protein expression is reduced. Furthermore, in the same tissues we also demonstrate that with a reduction in both GFPT1 and OGT, we see a reduction in the number of glycosylated proteins. This data suggests that FASN may not only regulate FA metabolism but also hexamine biosynthesis in APC/Villin-CRE mice.

To further elaborate on the role of GFPT1 and OGT in CRC tumorigenesis, we demonstrate here for the first time that inhibition of GFPT1 and OGT reduce cell proliferation and colony formation *in vitro*. Additionally, we show that knockdown of both GFPT1 and OGT reduced tumor volume and tumor weight in subcutaneous xenografts. These data further support the hypothesis that hexamine biosynthesis is an important metabolic pathway in the development and progression of CRC.

4.5 Conclusions

In summary, the APC/Villin-CRE mouse model in an excellent tool for the investigation of APC driven tumorigenesis [312]. Further investigation into the mechanistic drivers of this phenomenon could potentially provide new therapeutic avenues for the treatment of CRC. Our data here emphasizes the important role FASN plays in this process through the regulation of hexosamine biosynthesis. Furthermore, hexosamine biosynthesis itself appears to be a significant contributor to CRC progression and further understanding of this pathway is necessary for more efficacious treatment of CRC. Lastly, our data suggests that further understanding of the role FASN plays in APC driven tumorigenesis and targeting this association could lead to improved survival rates for patients with CRC.

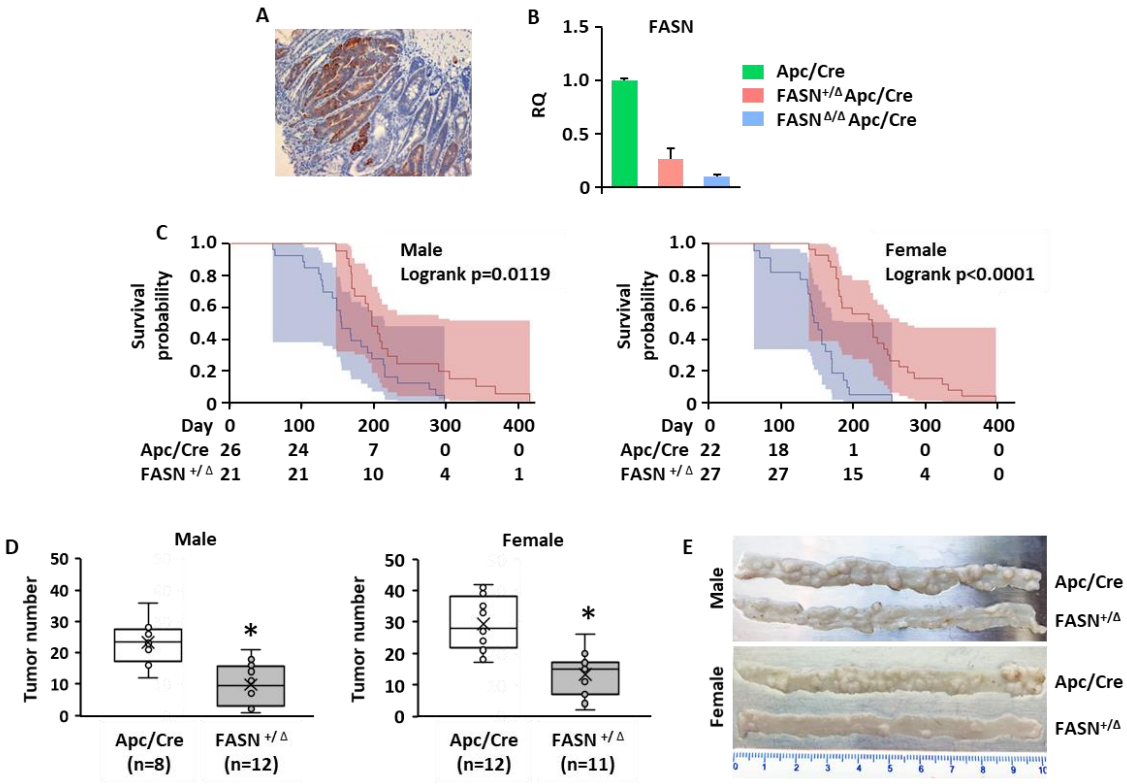


Figure 4.1 Heterozygous Deletion of FASN Increases Survival and Decreases Adenoma Burden *in vivo*.

(A) FASN expression in Apc/Cre mice. **(B)** Cre-mediated knockout of FASN in mouse intestinal tumors. **(C)** The effect of Villin-Cre-mediated heterozygous deletion of FASN in mouse intestinal and colon tissues on mice survival. **(D)** The effect of Villin-Cre-mediated heterozygous deletion of FASN in mouse intestinal and colon tissues on formation of mouse adenomas. Analysis10 cm sections of distal intestine from Apc/Cre mice and mice with heterozygous deletion of FASN.

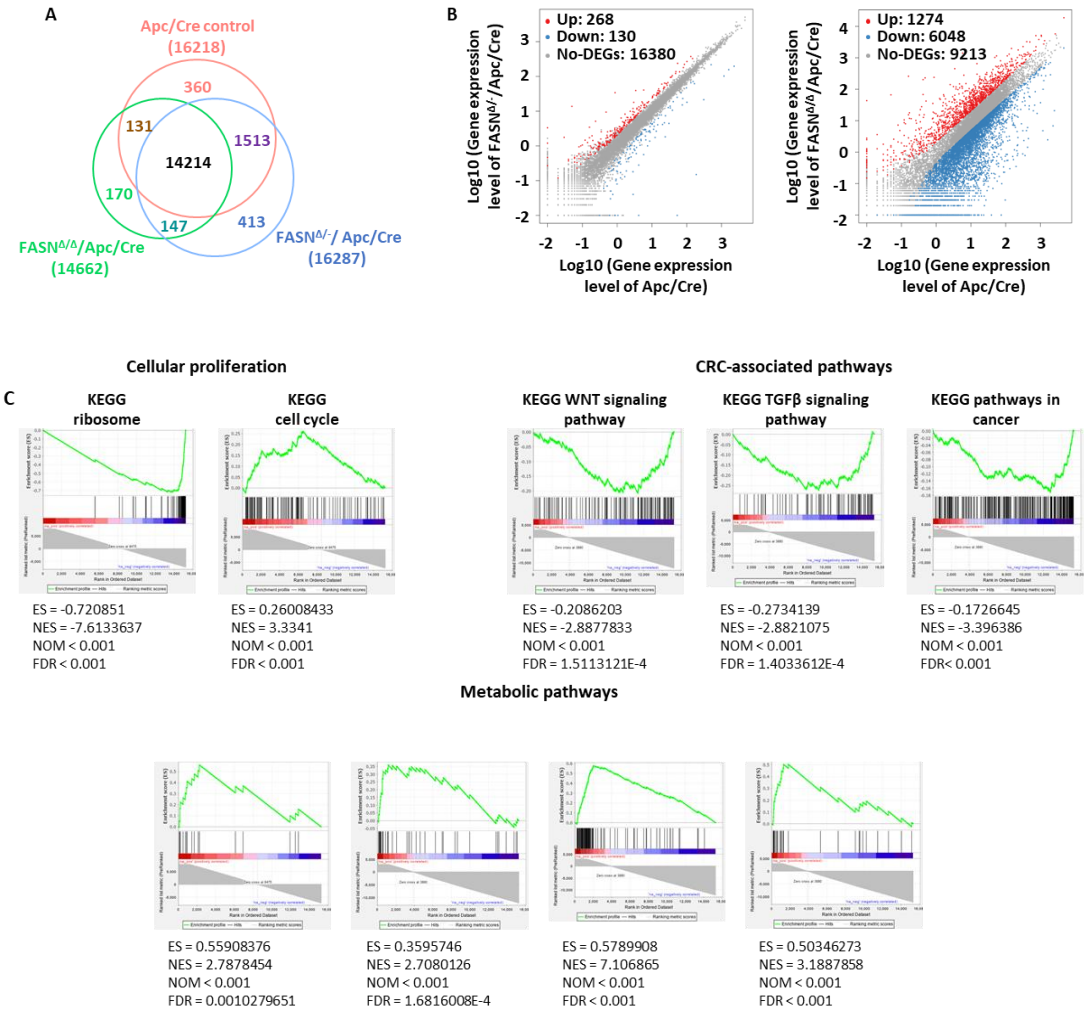


Figure 4.2 FASN Deletion Significantly Changes Cellular Gene Expression. **(A)** Venn diagram displaying the overlapping genes identified in tumors from Apc/Villin-Cre mice and Apc/Villin-Cre mice with heterozygous and homozygous deletion of FASN. **(B)** Scatter plots showing several DEGs in Apc/Villin-Cre mice and Apc/Villin-Cre mice with heterozygous and homozygous deletion of FASN. Significantly DEGs are indicated in colors. Red and blue dots are up- and downregulated genes, respectively. **(C)** Representative gene set enrichment analysis (GSEA) plots generated from RNA-seq expression data of Apc/Cre and FASN knockout mice. The bar codes indicate the location of the members of the gene set in the ranked list of all genes. ES, enrichment Score; NES, normalized enrichment score; NOM, nominal p-value; FDR, false discovery adjusted p-value.

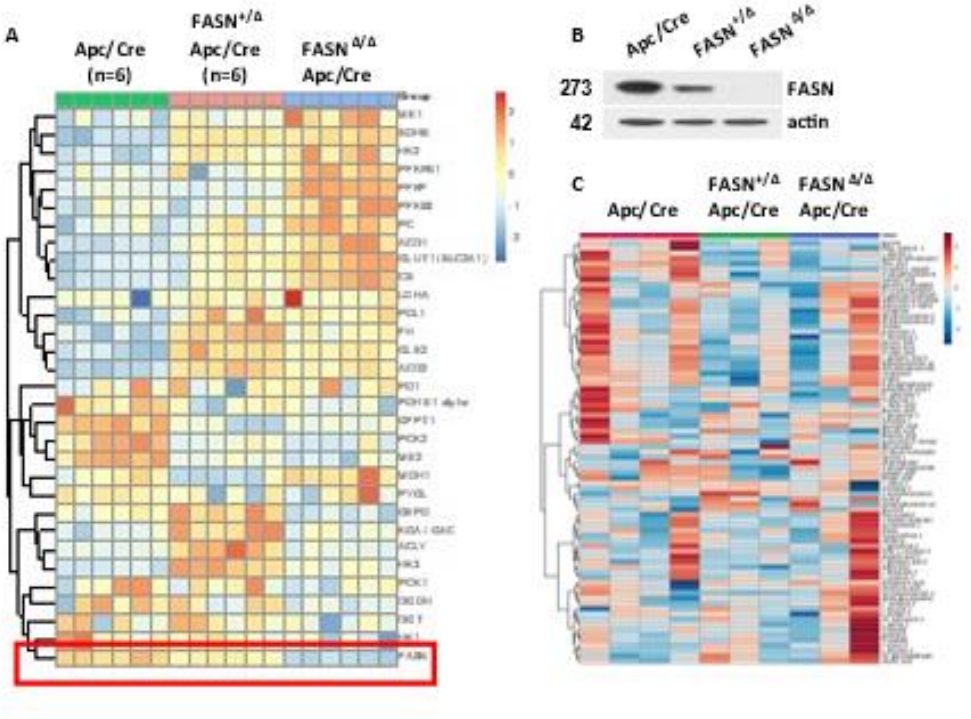


Figure 4.3 FASN Knockout Alters Cellular Metabolism.
(A) Heat map demonstrating expression of metabolic enzyme as determined by the Reverse Phase Protein Array (RPPA) analysis (performed by Redox Metabolism Shared Resource Facility and analyzed by Biostatistics & Bioinformatics Shared Resource Facility). **(B)** The level of FASN expression by Western Blot of mouse tumors used for the RPPA analysis. **(C)** Heat map of metabolites determined by Gas Chromatography-Mass Spectrometry (GCMS) in mouse adenomas (performed in collaboration with Dr. Ramon Sun laboratory).

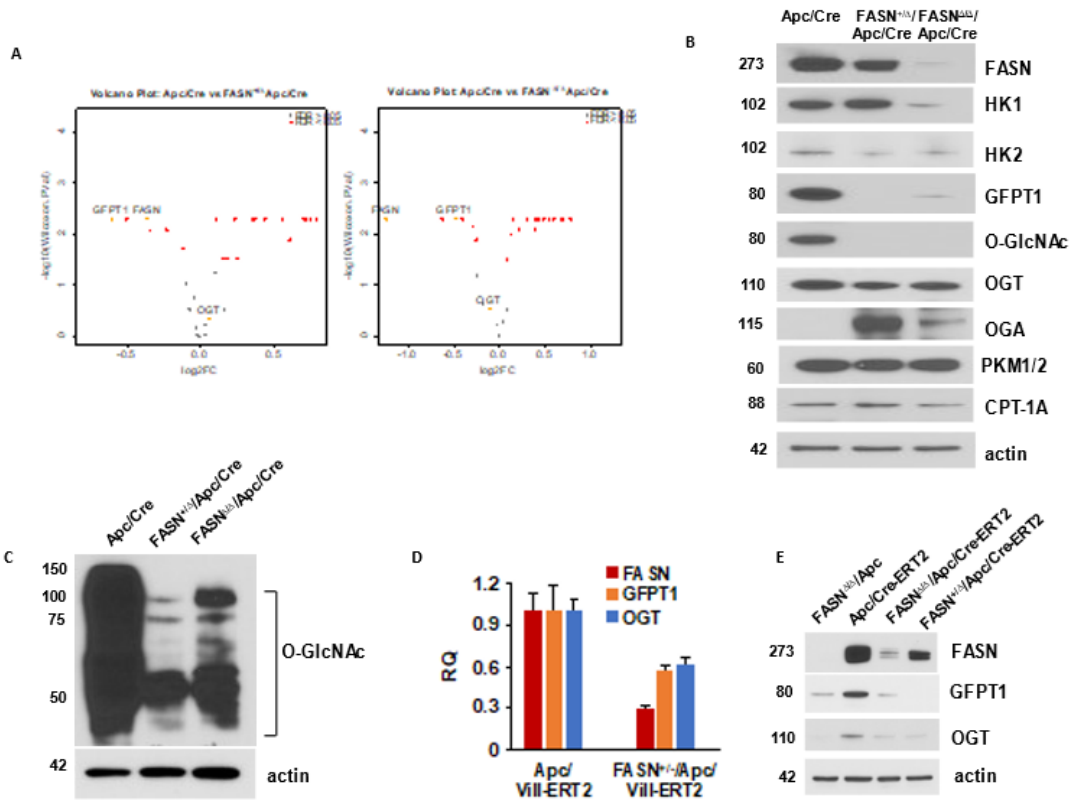


Figure 4.4 FASN Regulates the Expression of GFPT1 and OGT *in vivo*.
(A) RNA-seq analysis volcano plots showing significant differences in the mRNA expression of GFPT1 and OGT in FASN knockout mouse tissues. **(B)** Western blot analysis of various enzymes involved in hexosamine biosynthesis from Apc/Villin-Cre, $FASN^{+/\Delta}$ /Apc/Villin-Cre, and $FASN^{\Delta/\Delta}$/Apc/Villin-Cre tissues of mice. **(C)** Western blot analysis of O-GlcNAc in mouse tissues described above. **(D)** mRNA and **(E)** protein expression of FASN, GFPT1, and OGT in the inducible Apc/ Villin-Cre-ERT2, $FASN^{+/\Delta}$/Apc/ Villin-Cre-ERT2, $FASN^{\Delta/\Delta}$/Apc/ Villin-Cre-ERT2 mouse models.

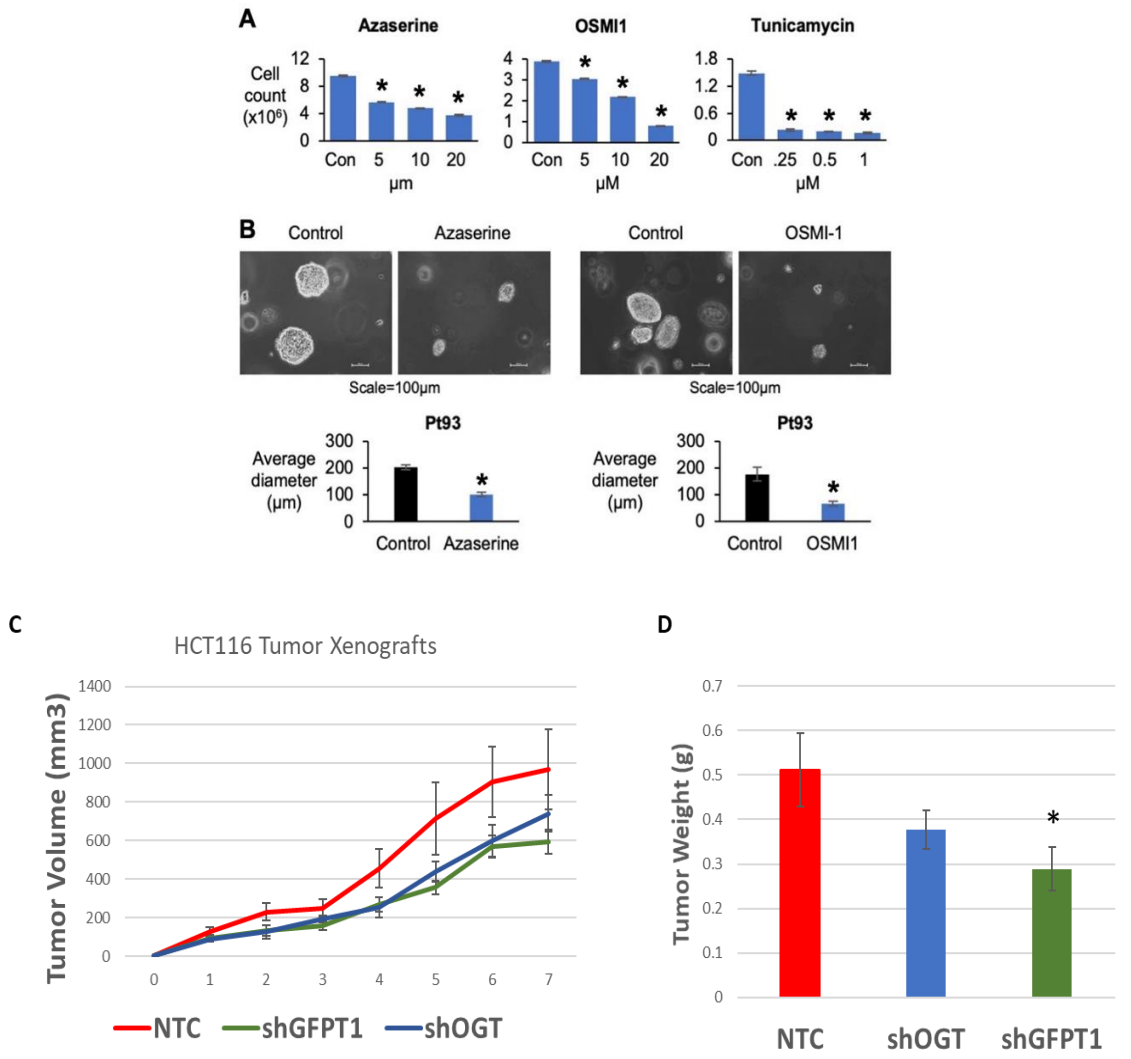


Figure 4.5 GFPT1 and OGT Regulate CRC Proliferation and Tumorigenesis **(A)** Cell proliferation assays of Pt93 cell treated with azaserine, and OSMI. **(B)** Colony formation assays of Pt93 cells treated with azaserine and OSMI-1. **(C)** HCT116 NTC, shGFPT1, and shOGT xenograft tumor volumes and **(D)** tumor weights.

CHAPTER 5. CONCLUSIONS

Altered fatty-acid metabolism is a hallmark of cancer and has been implicated in CRC progression and metastasis [128-130]. Inhibition of *de novo* FA synthesis through targeting FASN via TVB has shown some promise in pre-clinical studies [191-193]. However, despite of presence of high FASN expression in tumors, only 30% of PDXs show a positive response to TVB treatment [185]. Thus, there may be alternative compensatory pathways that contributes to TVB resistance. Such pathways may include the uptake of exogenous FAs via FA transporters. CD36 is a prominent exogenous FA transporter and has been shown to promote tumor progression in multiple diseases [149-152, 217-221]. Therefore, the purpose of my studies was to investigate any potential role of CD36 in CRC primary tumor progression and metastasis as well as any role CD36 may have in the resistance of FASN inhibition to possibly define new therapeutic targets for CRC treatment.

5.1 CD36 in Primary CRC

CD36 is upregulated in primary CRC cells, patient tissues, and patient derived xenografts. CD36 is associated with an increase in the proliferation of established and primary CRC cell lines. High expression of CD36 also promotes tumor growth *in vivo* in an isogenic patient derived xenograft model. Consistently, pharmacological inhibition of CD36 with SSO decreases cell proliferation *in vitro* and tumor progression *in vivo.* This data is in parallel with most of the current literature, which is suggestive that CD36 plays an important role in primary tumor survival and disease progression in multiple cancers [149-152, 217-221].

Furthermore, increased expression of CD36 was associated with increases in the pro-survival markers survivin and p-Akt. Survivin, a member of the inhibitor of apoptosis (IAP) family of proteins, is involved in the inhibition of various caspases including caspace-3 and 7, and thus inhibiting programmed cell death [328]. Akt is a crucial component of the PI3K/Akt/mTOR pathway, which is an important regulatory pathway for cell proliferation and survival [329]. This data is in agreement with previously published literature, which implicated CD36 in the regulation of the Src/PI3K/AKT/mTOR signaling pathway in HCC [221]. The data from this first study suggest that CD36 plays a role in proliferation and tumorigenesis in primary CRC.

During the investigative process of these findings, another manuscript studying the potential role of CD36 in CRC was published [330]. Interestingly, Y. Fang et.al. found that CD36 expression inversely correlates with CRC progression and they demonstrated that CD36 overexpression decreases CRC cell viability and colony formation [330]. Their data also suggest that CD36 knockdown increases cell viability and colony formation. Furthermore, they state that CD36 reduces glycolysis through inhibition of β-catenin/c-myc signaling [330]. This data is in contrast with previous literature in other diseases, which imply CD36 contributes

to increased glycolysis in HCC, and is in direct contrast with our findings here. One potential explanation to these varying results in CRC could be that between the two studies, a variety of CRC cell lines were used, none of which were used in both investigations. Our studies utilize HCT116, HT29 and isogenic HT29 cell lines LuM0 and LuM3, as well as primary cell lines established from PDX tumors in our laboratory. We also utilized a variety of patient tissues and other PDX models [274]. In contrast, Y. Fang et al. used 4 established CRC cell lines: SW480, LoVo, RKO, and CACO2 [330] and did not utilize HCT116 and HT29 cells which are most frequently used in publications for CRC. Even among the few CRC cell lines used within our investigation, we noted differences in the expression of CD36. Variances in the mutational profiles, metabolic reprogramming, and gene expression of these cell lines may account for the conflicting results observed between the two manuscripts.

5.2 CD36 Compensates for FASN Inhibition

Previous studies from our lab have shown that inhibition of FASN via TVB-3664 reduces cell proliferation *in vitro* [153, 185]. However, when analyzing the efficacy of TVB in pre-clinical *in vivo* studies using PDX models, we noted that only 30% of PDX tumors responded positively to TVB-3664 [153, 185]. Since mice consume a dietary FAs present in their normal chow, we hypothesized that pathways involved in the uptake of exogenous FAs are of particular interest to investigate the mechanisms of resistance to FASN-targeted therapy.

CD36 is a well-defined FA transporter expressed in various tissues [120]. Analysis of the TCGA data base reveals that on the mRNA level, CD36 and FASN share an inverse relationship in CRC [274]. In the presence of FASN inhibition or knockdown, we show that CD36 is significantly upregulated in established and primary cell lines, tumor xenografts, genetically modified mouse models with knockout of FASN, and human tissues [274]. Furthermore, this upregulation of CD36 is extremely specific, with no significant change in the expression of other prominent FA transporters when FASN function is reduced in all models described above [274]. Additionally, knockdown of FASN upregulates the uptake of exogenous fluorescent FA analogues, and this increase in FA uptake is directly dependent on CD36 as demonstrated using a CD36 neutralizing antibody [274]. Together, the data presented here provides compelling evidence that inhibition of FASN directly upregulates CD36 to increase exogenous FA uptake and compensate for the lack of *de novo* FA synthesis. This relationship between FASN and CD36 had not yet been investigated in CRC.

Interestingly, our data here is further supported by one other study. It was shown that breast cancer cell lines which are resistant to lapatinib, a targeted therapeutic for HER2 positive breast cancer cells, markedly develop resistance to FASN inhibition [331, 332]. To note, it has been shown that FASN can be regulated by HER2 via phosphorylation in HER2 positive breast cancer [333]. It was suggested that metabolic reprogramming of these cells contributes to their

resistance to both lapatinib and FASN inhibition. Indeed, it was shown through microarray analysis that CD36 is significantly upregulated in lapatinib resistant breast cancer cell lines and that inhibition of CD36 significantly reduces cell viability and proliferation [331]. Furthermore, inhibition of CD36 via a neutralizing antibody in mouse xenograft models sensitizes breast cancer tumors to lapatinib [332]. Lastly, it was also demonstrated that patients treated with anti-HER2 therapy display a significant upregulation in CD36 expression [332]. Together, these data support our findings in that, with the targeting of HER2 and thus FASN, breast cancer cells reprogram their metabolism to become more reliant on the uptake of exogenous FAs through CD36.

Due to the increasing interest in targeting FA metabolism in the treatment of cancer, understanding the relationships between exogenous FA uptake and *de novo* FA synthesis is critical. From the data presented here, we suggest that further understanding of the relationship between FASN and CD36 is necessary to increase the efficacy of FASN-targeted therapies as well as those drugs already approved for the treatment of other cancers, such as lapatinib. Furthermore, delineating the mechanisms behind this relationship may help to develop entirely new therapeutic approaches for the treatment of CRC, other cancers, or other metabolism dysregulated diseases.

5.3 CD36 and CRC Metastasis

TMA analysis of normal colon as well as primary and metastatic CRC tissues showed an increase in CD36 expression in primary CRC and further upregulation in metastasis [274]. Furthermore, CD36 expression in metastatic tissues could be characterized with a stark increase in membrane associated CD36 [274]. Because of this association of CD36 expression with metastatic tissues, we developed a hypothesis that CD36 expression contributed to CRC progression and metastasis.

Indeed, CD36 overexpression in CRC cell lines increased cell invasion and colony formation *in vitro* and CD36 expression was associated with cells that exhibit a higher metastatic potential [274]. Furthermore, CD36 knockdown reduced lung colony formation and CD36 overexpression increased local CRC cell invasion and metastasis in cecum injected mice [274]. It is logical to think that increased CD36 expression in CRC confers metastatic potential through increased FA uptake and, thus, more energy resources and building blocks for cell membranes in these more metabolically demanding and highly proliferative cells. However, it is possible that CD36 may also regulate other processes involved in metastasis, such as EMT.

We found that CD36 expression is associated with the newest member of the matrix metallopeptidase family of proteins, MMP28 [290, 292]. We also show that knockdown of MMP28 reduces CRC cell invasion comparable to that of CD36 knockdown. Current literature on the details of MMP28 function are limited due to

its novelty; however, like other MMPs, it is implicated in the regulation of various extracellular matrix components [290]. Most interestingly, we found that MMP28 expression is inversely associated with e-cadherin expression, an important marker for EMT in CRC [284]. As previously mentioned, e-cadherin loss is a poor prognostic factor for CRC patients, and it is associated with tumor invasion and lymph node metastasis [287]. It was previously shown that MMP28 is associated with EMT and e-cadherin loss in lung carcinoma [295, 296]. However, any potential association of MMP28 with e-cadherin has not yet been investigated. Moreover, the exact mechanism by which MMP28 may regulate the expression of e-cadherin has not yet been fully elucidated. We demonstrate here, for the first time, that MMP28 expression is not only associated with loss of functional e-cadherin, but that it is also associated with an increase in the products of e-cadherin cleavage, CFT1 and CFT2 [297]. This striking insight suggests that MMP28 may very well directly cleave functional e-cadherin. The implications of these findings are significant, as the CD36-MMP28-e-cadherin association may be a novel mechanistic pathway of EMT regulation in CRC.

With metastatic CRC survival rates remaining at terribly low levels, insight into mechanisms by which CRC tumors initiate metastasis are crucial for future treatment of CRC patients. Further investigation into the regulation of MMP28 by CD36 may provide the necessary information to successfully develop therapeutics against this phenomenon.

5.4 CD36 Functions as a FA Transporter in Cancer

Mechanistically, we show an increase in exogenous FA uptake as well as increases in both survivin and p-Akt and as described above, which mostly agrees with previous literature with some slight controversy [330]. The results here lead to the development of the hypothesis that CD36 enhances primary CRC proliferation and survival directly through the uptake of exogenous FAs, and thus increased FA metabolism. However, further investigation into exact metabolic pathways which are utilized in cells and tumors with high expression of CD36 is needed to definitively test this hypothesis.

Compelling evidence exists from multiple previous publications that CD36 plays an important role in tumorigenesis and cancer progression via FA transport [219, 220, 276]. As previously mentioned, it was shown that FA treatment of breast cancer cell lines enhances cell proliferation. Furthermore, knockdown of CD36 abolishes cell proliferation after FA treatment, suggesting that the more proliferative phenotype observed with FA treatment is CD36 dependent. [219]. In addition to breast cancer, CD36 was shown to contribute to cervical cancer tumor growth and progression in a FA dependent manner. More specifically, cervical cancer xenograft mice fed a diet high in oleic acid exhibit increased tumor volume and weight [276]. However, when CD36 is inhibited, the tumorigenic phenotype observed in oleic acid fed mice is prevented. Furthermore, overexpression of CD36

in cervical cancer xenograft mice, which were fed normal diet, results in similar tumor growth as just high oleic acid diet alone [276].

Interestingly, the pro-tumorigenic effect of CD36 as a FA transporter are not strictly due to just the transport of various FAs. In fact, it was shown that the binding and transport of FAs by CD36 also induces several downstream signaling cascades which also contribute to disease progression [210, 221, 276]. For instance, in cervical cancer cells, CD36 FA transport activates ERK 1/2 signaling through activating Src-kinase, as described previously [210, 276]. Furthermore, CD36 was found to enhance glycolysis in HCC through downstream activation of the Src/PI3K/AKT/mTOR signaling pathway [221]. Our data suggest that a similar signaling function is also present in CRC, as we show an increase in both survivin and p-Akt when CD36 is overexpressed in CRC cells.

Our results support the literature in that CD36 promotes CRC through the uptake of exogenous FAs and the upregulation of multiple pro-survival pathways. Further investigation into the exact metabolic rewiring that occurs due to CD36-mediated FA uptake is needed and would likely shed new light on FA metabolism as a therapeutic target in CRC. Together, our data and the literature suggest that targeting CD36 from the perspective of a FA transporter may yield significant insight into this phenomenon and possibly provide new therapeutic strategies for CRC and other oncogenic diseases.

5.5 Potentiality of CD36 Targeted Therapies

As mentioned previously, there were multiple clinical trials which had targeted CD36 in relation to its role in binding TSP1 and its role in angiogenesis [226]. More specifically, these therapeutics were designed as TSP1 mimetics, mimicking the structure of TSP1 to further increase TSP1-CD36 mediated signaling and, thus, inhibiting angiogenesis [208, 209, 226]. Although each drug had shown promise in pre-clinical studies, unfortunately, each of those previous clinical trials resulted in early termination due to adverse side effects, as well as little to no efficacy [227]. To date, there have been no pre-clinical or clinical trials developed aimed at targeting CD36 in cancer from the perspective of FA transport.

As the studies presented here suggest, targeting CD36 can both reduce CRC cell proliferation and inhibit xenograft tumor growth *in vivo*. Additionally, CD36 contributes significantly to invasion and colony formation *in vitro*, and lung colony formation and orthotopic xenograft metastasis *in vivo,* suggesting that it is a potential target for metastasis. Furthermore, we show that inhibiting CD36 function directly decreases exogenous FA uptake, supporting the hypothesis that CD36 promotes primary and metastatic CRC through the advantageous uptake of exogenous FAs which can then be used as a source of energy as well as building blocks for cell membranes [274]. These attributes strongly support the idea of developing and further investigating the effectiveness of chemotherapeutic targets against CD36, particularly in its role as a FA transporter.

Previous investigations into the role of CD36 in oral carcinoma revealed that CD36 is a major contributor to oral carcinoma metastasis [150]. Interestingly, in contrast to our findings in CRC, this study shows little to no effect on primary oral carcinoma tumor growth when CD36 was inhibited or knocked down [150]. Because of these earlier findings, members from this lab have since founded a company, Ona Therapeutics, which is focused on the investigation of metastasis in numerous oncogenic diseases with the primary aim of developing chemotherapeutics against dysregulated lipid metabolism **(Figure 5.1)** [334]. As their flagship project, CD36 is currently their primary focus with the hopes of developing agents to inhibit metastasis initiation in various cancers. Our work presented here shows substantial evidence supporting their current work and suggests that CD36 is a potential therapeutic target in CRC, not only for metastasis but also for primary CRC development and progression.

To reiterate the potential significance of CD36 as a therapeutic target, I will stress again the important role CD36 plays in the compensation of *de novo* FA synthesis inhibition. Currently, the results described here regarding this relationship could potentially have significant clinical impact. The FASN inhibitor TVB and its various analogues are being tested in several pre-clinical and clinical trials [191-193]. Current data not only suggests that not all patients will respond well to FASN-targeted therapy, but also that high expression of FASN alone is not enough to determine whether a patient will respond positively to TVB. Identifying CD36 as a potential compensatory mechanism to FASN inhibition may allow for more efficacious treatment in combination with TVB.

Chemotherapeutic targets against CD36 should be developed based on our results and the overwhelming evidence of CD36 as pro-tumorigenic in the literature. Together, the results shown here support the need for further investigation of CD36 to treat CRC more effectively. This further understanding will allow investigators, such as those working at Ona Therapeutics, to better develop potential drugs to target CD36.

5.6 FASN Promotes APC-Driven Tumorigenesis

FASN expression is upregulated in animal tissues with Cre-mediated deletion of the APC gene. Heterozygous deletion of FASN in Apc/Villin-Cre mice leads to a significant increase in animal survival and a decrease in intestinal tumor burden. This data suggests that FASN may play an important role in tumor initiation in APC-driven tumorigenesis.

Investigations in the molecular drivers for both familial and sporadic CRC have resulted in several suspect genes. One such is APC, and mutations in APC and its role in regulating Wnt/β-catenin signaling, have been shown to be necessary for the initiation of FAP, and aberrant Wnt/β-catenin signaling is present in nearly 90% of human CRC [309, 310, 335]. The *in vivo* APC/Villin-Cre mouse

model is an excellent tool for investigating this phenomenon [312]. Our data here suggests that not only is APC driving tumorigenesis through increased Wnt/β-catenin signaling, but lack of wild-type APC also significantly upregulates FASN and FASN itself is a major contributor to the pro-tumorigenic phenotype seen in the APC/Villin-Cre mouse model.

To further investigate potential mechanisms behind FASN dependent tumorigenesis, we analyzed these mouse tissues for global mRNA using RNAseq analysis as well as performed metabolic analyses. Interestingly, we found that FASN significantly regulates a spectrum of cellular processes. These include cell cycle, cell proliferation, and multiple metabolic pathways. More specifically, we found that FASN is associated with an increase in glycolysis and hexosamine biosynthesis (glycosylation), with emphasis on the expression of two prominent enzymes associated with glycosylation, GFPT1 and OGT. Upon further investigation of the role these two enzymes might play in CRC, we showed that OGT and GFPT1 themselves contribute to CRC proliferation, colony formation, and tumorigenesis *in vivo*. This data suggests that FASN may help contribute to APC-mediated CRC initiation through enhanced hexosamine biosynthesis and altered glycosylation. Based on this data, our lab has been further investigating the contribution of FASN to O-linked and N-linked glycosylation.

FASN, as previously mentioned, is already identified as a potential therapeutic target for the treatment of CRC and several clinical trials are currently underway using the FASN inhibitor TVB [191-193]. The data here suggests that treatments targeting FASN may prove more effective if the CRC tissues contain a mutation in APC. Additionally, targeting FASN in combination with hexosamine biosynthesis may yield even greater treatment efficacy. Thus, we suggest that with further investigation into FASN mediated tumorigenesis in CRC, from the perspective of alterations in metabolic processes other than direct FA metabolism, more effective treatment strategies for CRC may be developed.

5.7 Future Directions

Through a large variety of *in vitro* and *in vivo* methods as well as in human tissues, we demonstrate a significant and specific upregulation of CD36 in the presence of FASN inhibition or knockdown. However, the mechanistic drivers of this upregulation have yet to be delineated. There are multiple upstream pathways and effectors that can act on either FASN, CD36, or both. Both genes contain sequences within their promoter regions which can be targeted by the sterol-regulatory element binding proteins (SREBPs) as well as PPARs and STAT proteins [200, 202, 336]. It is possible that in the absence of functionally *de novo* lipid synthesis, one or some of these regulatory pathways and transcription factors are upregulated in an attempt to compensate, leading to an increase in CD36 expression and an increase in exogenous FA uptake. In addition to transcriptional regulation of CD36, it is worth noting that we observed an increase in membrane bound CD36 when FASN was inhibited or knocked down. This suggests that there

may be some post-transcriptional regulation of CD36 involved when *de novo* lipid synthesis is reduced. As previously mentioned, palmitoylation of CD36 and the presence of di-sulfide bridges within the protein are critical for subcellular trafficking of CD36 the cell membrane and its association with lipid rafts [194]. Further investigation to the post-transcriptional regulation of CD36 when functional FASN is reduced is necessary to fully delineate the association between these two proteins that we describe in Chapter 2.

In addition to understanding the mechanistic link between CD36 and FASN, further analysis of the translational effect of combining the inhibition of these two proteins is necessary. We show that when we inhibit both FASN and CD36, we see a combinational effect on decreased cell proliferation in multiple established and primary cell lines. This *in vitro* data will need to be further elaborated on, by transitioning to *in vivo* models with combinational treatment of both TVB and a CD36 inhibitor. We reported in Chapter 2 that *in vivo* administration of SSO, an inhibitor of CD36, significantly decreases subcutaneous xenograft tumor growth but has no significant effect on animal body weight. This is encouraging and suggests that a combination administration of both TVB and SSO may be possible. There are companies actively developing inhibitors against CD36 with clinical intent, such as Ona Therapeutics [334]. It is a possibility that if SSO proves to be too toxic when used in combination with TVB *in vivo*, that novel inhibitors may prove useful in such an investigation.

Although we demonstrate the important role CD36 plays in the proliferation, tumorigenesis, and metastasis of CRC, more work is needed to delineate exact mechanisms responsible for this phenomenon. One potential mechanism is the direct uptake of exogenous FAs in the presence of increased CD36 expression, leading to increased FA metabolism. We show in Chapter 2 that with increases in CD36 expression, we observe an increase in the uptake of exogenous FA analogues. However, the specific types of FAs up taken by CD36, and their fate are not known.

There are several methods utilized today to study various metabolic pathways which are enhanced in cancers cells. The cutting edge of these methods is known as carbon-13 (^{13}C) metabolic flux analysis [337]. This method involves the labeling of a particular metabolic substrate, such as glucose or FAs, with a stable isotope of carbon, such as ^{13}C. When these molecules are metabolized by the cell, the downstream metabolites are consequently labeled in a unique pattern by the isotopes, which can be measured using techniques such as mass spectrometry (MS) or nuclear magnetic resonance (NMR) [337]. Metabolic tracing studies utilizing ^{13}C labeled long-chain FAs of varying length could yield new insights into the types of FAs CD36 uptakes in CRC and ultimately how those FAs are utilized. Furthermore, Seahorse analysis of extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) on CRC cell lines utilized in Chapter 2-3, such as the CD36 knockdown and overexpression cell lines, is necessary to determine if uptake of extracellular FAs via CD36 alters FA β-oxidation and

glycolysis. These investigations will be critical in determining the role CD36 plays in CRC tumorigenesis via direct increased exogenous FA uptake.

In addition to increased FA uptake and potentially an increased in FA metabolism, we also observed as association between CD36 expression and that of p-AKT and survivin. However, mechanistic links between CD36 expression and increases in both survivin and p-AKT have not yet been delineated. Recently, it has been shown that palmitic acid (PA) alone induces migration and invasion of gastric cancer cells in a CD36 dependent manner [338]. Thus, PA treatment studies in combination with altered expression or inhibition of CD36 in CRC could determine if the increased activation of the AKT pathway and an increase in survivin expression are directly related to exogenous PA up taken by CD36.

Aside from the pro-tumorigenic and proliferative properties of CD36 in CRC that we describe here, we also demonstrate that CD36 significantly promotes CRC metastasis. We showed that CD36 expression is associated with the newest member of the matrix metallopeptidase family of proteins, MMP28, and that MMP28 is associated with cleavage of e-cadherin. However, any direct mechanistic link between CD36 expression and that of MMP28 remains elusive. Being the most novel member of this family of proteins, resources on the MMP28 gene are limited, however, the gene has been sequenced. Other members of the MMP family have promoter sequences for tumor necrosis factor alpha (TNFα) as well as binding sites for NF-kB, signal transducers and activators of transcription (STAT) proteins downstream of EGF signaling, and TGFβ [339-341]. Previous studies in hepatocellular carcinoma have demonstrated that FFA treatment promotes EMT in a CD36 dependent manner through increased activation of TGFβ signaling [222]. In depth analysis of the promoter region of the MMP28 gene sequence is necessary to identify potential enrichment sites for promoters, such as TGFβ, that could potentially link MMP28 and CD36 mechanistically. Such future investigations will shed new light on CD36 and its role not only in CRC and CRC metastasis, but potentially yield new insight into roles CD36 may play in other diseases.

Lastly, we show here that FASN promotes APC-driven tumorigenesis in CRC and does so through the regulation of hexosamine biosynthesis. Further investigation into the potential of targeting both *de novo* lipid synthesis and hexosamine biosynthesis in combination will need to be performed both *in vitro* and *in vivo*. Additionally, because of the significant reduction in tumor burden observed in mice with FASN heterozygous knockout, it is possible to target FASN via TVB in the APC/Villin-Cre mouse model to further investigate this phenomenon. Analysis of current patient data basis for the expression patterns of both FASN and mutant APC will need to be performed and could identify potential correlations between these two genes and CRC patient prognosis. Together, the data presented here and that of future investigations described could further delineate the exact role FASN plays in CRC tumorigenesis and progression.

5.8 Final Thoughts

CRC patients with distant metastatic disease are still in desperate need for new therapeutic interventions. Stage IV CRC patients have severely low 5-year survival rates and disease relapse is prominent amongst most patients. Investigation into altered molecular and metabolic pathways that drive CRC progression and metastasis is necessary to develop more effective treatments. Altered FA metabolism is emerging as a prominent target for the treatment of CRC, and studies from our lab have already demonstrated some of the importance that *de novo* FA synthesis via FASN plays in CRC progression and metastasis. The results of the studies described here also identify CD36, a prominent FA transporter expressed in CRC, as a new potential therapeutic target for this devastating disease. We further emphasize the striking relationship between FASN and CD36, especially the upregulation of CD36 when FASN is effectively inhibited. This is particularly important as TVB inhibitors against FASN are currently in clinical trials. Aside from this compensation, we also describe the pro-tumorigenic and metastatic properties of CD36 in the regulation of pro-survival markers and e-cadherin cleavage via MMP28 and suggest that targeting CD36 could yield positive clinical results in CRC. Furthermore, we identified other pro-tumorigenic properties of FASN, as shown using the APC/Villin-Cre mouse model and how FASN can regulate cellular metabolism, particularly, hexosamine biosynthesis.

Together, the data from the studies presented here offer new insights into the complex regulation of FA metabolism in CRC. Further investigation into mechanistic drivers behind CD36-mediated CRC progression and metastasis are necessary to develop new chemotherapeutics. Additionally, further understanding of CD36's role in the compensation of FASN inhibition and the regulation of glycosylation by FASN, could drastically improve the treatment efficacy of the clinical trial drug TVB. Cumulatively, these studies show significant promise of targeting lipid metabolism in the future development of treatment strategies for CRC and beyond.

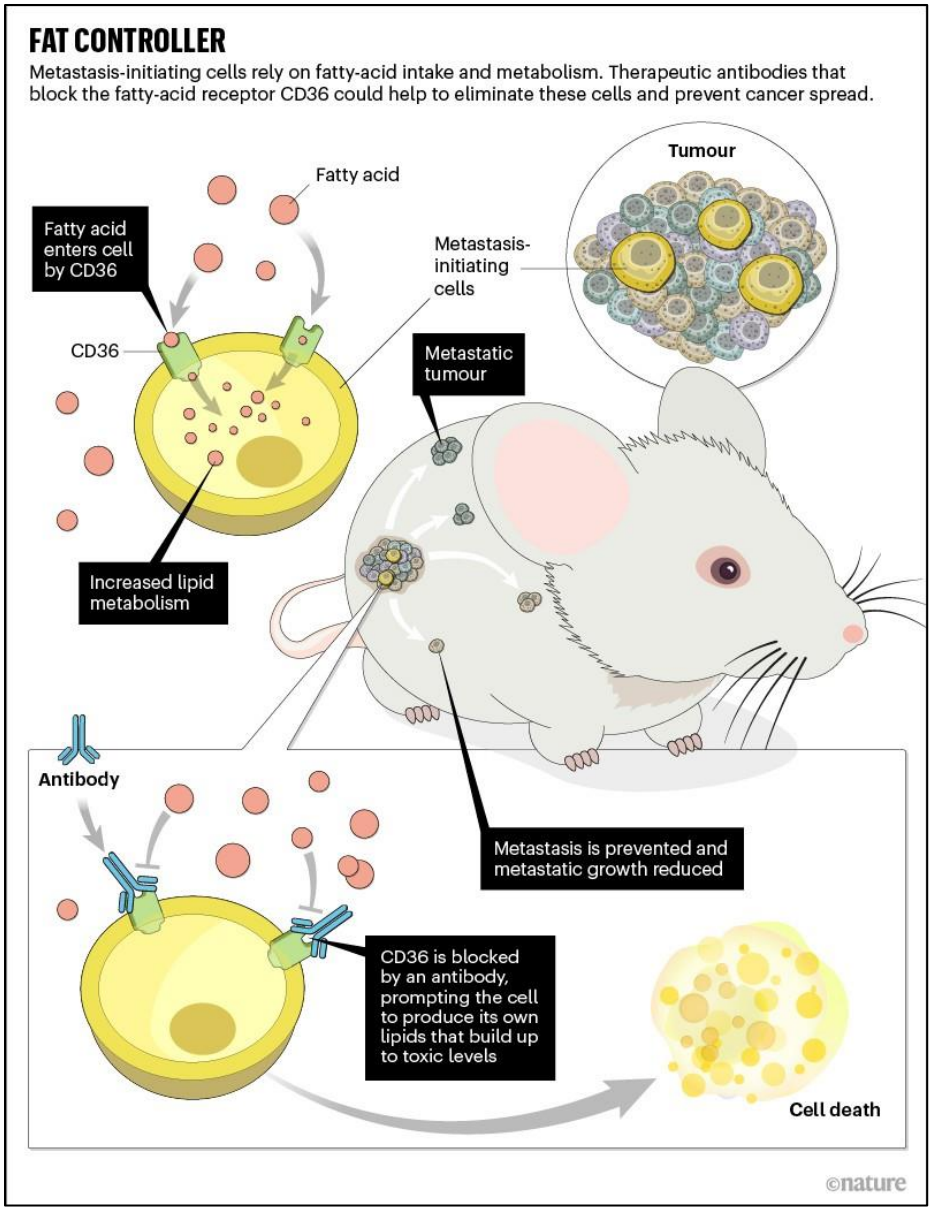


Figure 5.1 Targeting Metastasis via FA uptake.
Schematic showing antibody targeting of CD36 as a potential therapeutic model in cancer metastasis as described by Ona Therapeutics [334].

ACKNOWLEDGEMENTS

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APPENDICES

APPENDIX 1. METHODS FOR CHAPTER 2

Colon Cancer Established and Primary Cell Lines

Established cell lines HCT116, HT29, and HT29LuM3 were maintained in McCoy's 5A medium supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO) and 1% penicillin–streptomycin. Primary colon cancer patient Pt 93 and Pt 130 cultures were isolated and established from PDX tumors as previously described (1). Cells were maintained as monolayer culture in DMEM supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO) and 1% penicillin–streptomycin. Primary Pt 93 and Pt 130 colon cancer cells were authenticated as unique human cell lines (Genetica). Established CRC cell lines were authenticated using STR DNA profiling (Genetica, Cincinnati, OH). Stable CD36 knockdown HCT116, HT29, and HT29LuM3 cell lines were established using CD36 shRNAs from Sigma-Aldrich (TRCN000005699, TRCN0000057000, and TRCN0000057001). Cells were selected with 10mg/mL puromycin. Knockdown was confirmed via quantitative real-time PCR (qRT-PCR) after cell selection and prior to performing animal experiments. Overexpression cell lines were established by transfecting HCT116 cells with either pCMV-Spark-CD36 (Sino Biological Inc. NM 001001547.2), td-Tomato-CD36 (Addgene, Plasmid #58077).

Tissue Microarray Analysis-Immunohistochemistry

Immunoreactivity scores of CD36 and FASN expression were analyzed in matched normal colon mucosa and tumor tissues from patients diagnosed with Stage I-IV CRC who had surgery at UK Chandler Medical Center (TMA ID BH15991A, n = 56) by a GI pathologist (EYL) blinded as to tumor stage. The final immunoreactivity score was determined by multiplication of the values for staining intensity (0, no staining; 1, weak; 2, moderate; 3, strong staining) and the values for percentage of positive tumor cells (0, no positive cells; 1, 0–10%; 2, 11–50%; 3, 51–100% positive).

Tissue Collection

Tissues were obtained from consented patients with Stage II-IV CRC who had undergone surgery at UK Medical Center (IRB #16-0439-P2H). 6–8-week-old NSG mice (NOD.Cg-Prkdc Il2rg /SzJ) from The Jackson Laboratory (Bar Harbor, ME) were used for PDX models. All procedures were performed using protocols approved by the UK Animal Care and Use Committee. Briefly, CRC tissues (2–5 mm) obtained from CRC patients of both sexes were implanted subcutaneously into their flanks in a small pocket surgically created under the skin. Established tumors were designated as generation 0 (G0). Tumor tissues from G0 were minced and mixed with Matrigel to ensure homogeneous distribution of tissues among mice and allow implantation of an equal volume of tumor tissues into the flank. Tumor tissues were resected when they reached an appropriate size and digested as previously described [1]. For evaluation of CD36 expression in PDX models, we utilized tissue samples from Pt 2402 PDX model established from a patient diagnosed with metastatic adenocarcinoma (lung) consistent with colon primary tumor [1]. Pt 2402 PDX tumors were grown to approximately 200 mm^3 then tissues were collected and lysed for analysis via western blot.

Flow Cytometry

Individual cells from PDX model Pt 2402 were stained for CD36 with fluorescent antibody (Abcam ab23680). Stained cells were sorted via flow cytometry and the top 10% of GFP positive CD36 expressing cells ($CD36^{high}$) and the bottom 10% of GFP negative cells ($CD36^{low}$) were sorted separately from the rest of the tumor cell population. $CD36^{high}$ and $CD36^{low}$ were mixed with 100 µL of 30% Matrigel and subcutaneously injected into NSG mice. Tumor growth was monitored for three months. Samples were taken from subsequent tumors for western blot analysis and immunohistochemistry and the remaining tumor tissue was re-sorted for CD36.

FA Uptake

HCT116, NTC and FASN shRNA, cells were plated at 10,000 cells/well on an 8-well coverslip u-slide (Ibidi #80826) and treated with CD36 neutralizing antibody (Cayman Chemical #1009893) for 24 hrs. After incubation with neutralizing antibody, cells were then treated with fluorescent FA analogue BODIPY FL (Thermo Fisher #D3822) for 10 minutes in serum free McCoy's 5A medium supplemented with 10% FA free BSA. Cells were washed twice with PBS and fixed with PBS containing 5% formalin for 20 minutes at 37C. Cell were then imaged via confocal microscopy using a Nikon A1 Confocal Microscope.

Cell Proliferation Assay

CRC cell lines were plated onto 24 well plates at a concentration of 30,000 cells per well. Cells were given DMEM medium for Pt 93 and Pt 130 and McCoy's 5A medium for HCT116 with and without fetal bovine serum to simulate starvation conditions. Cells were also treated with or without 100 µM SSO (Cayman Chemical) or 0.2 µM TVB-3664 or both. TVB-3664 was provided by Sagimet Biosciences (Menlo Park, CA).Cells were incubated at 37°C for 6 days. After the incubation period, cells were trypsinized and collected individually based on well and condition of treatment. Cells were counted using a Vi-Cell XR Cell Viability Analyzer (Beckman Coulter #C19196). HCT116, NTC and CD36 shRNA (#2 and #4), cells were plated onto 24 well plates at a concentration of 30,000 cells per well. Cells were cultured in McCoy's 5A medium with and without fetal bovine serum for 72hr and counted as described above.

Quantitative Real-Time PCR

Total RNA was isolated using an RNeasy mini kit (QIAGEN). cDNA was synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystems). QRT-PCR was carried out using a TaqMan Gene Expression Master Mix (#4369016) according to manufacture protocol and TaqMan probes for human CD36 (ID Hs00354519 m1), human FASN (ID Hs01005622 m1), human FATP3 (ID Hs00354519 m1), human FATP4 (Hs00192700 m1) and human GAPDH (#4333764F; Applied Biosystems).

Subcutaneous Xenografts

NU/NU mice were injected subcutaneously with 1.0×10^6 cells of HCT116 NTC (non-targeted control, n=7), shCD36 #2 (n=6) or shCD36 #4 (n=7) in 100 µL PBS and tumor growth was monitored. Tumor size was measured via calipers every three days and tumor volume was calculated using the formula: TV = width^2 x length / 0.52. When NTC tumor growth reached approximately 200 mm^3, all mice were sacrificed, and tumor weight was taken via digital scale. NU/NU mice were injected subcutaneously with 2.0×10^6 cells of NTC (n=5) and shCD36 #4 (n=5) in 100 µL PBS for HT29 and HT29 LuM3 xenografts experiments.

Genetically Modified Mice

C57BL/6J mice with LoxP-flanked FASN alleles were obtained from Clay Semenkovich, MD at Washington University, and FASN/VillinCre and FASN/Apc/VillinCre mouse colonies were established by mating these mice with C57BL/6J Villin/Cre and C57BL/6J Apc/Cre mice in Dr. Zaytseva's laboratory [128].

APPENDIX 2. METHODS FOR CHAPTER 3

Colon Cancer Established and Isogenis Cell Lines

Established cell lines HCT116, HT29, and HT29LuM3 were maintained in McCoy's 5A medium supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO) and 1% penicillin–streptomycin. HT29 LuM0 and LuM3 cell lines were established as previously described [254]. Stable CD36 knockdown HCT116, and HT29LuM3 cell lines were established using CD36 shRNA from Sigma-Aldrich (TRCN0000057001). Cells were selected with 10mg/mL puromycin. Knockdown was confirmed via quantitative real-time PCR (qRT-PCR) after cell selection and prior to performing *in vitro* and *in vivo* experiments. Overexpression cell lines were established by transfecting HCT116 and HT29 LuM0 cells with either pLenti-C-Myc-DDK-P2A-Puro-CD36 overexpression (OriGene # RC221976L1V) or pLenti-C-Myc-DDK-P2A-Puro-Empty-Vector (OriGene #PS1000064) lentiviral transduction particles. MMP28 knockdown cell lines were generated by transfecting HCT116 cells with siMMP28 siRNA (Invitrogen #87705767) or scrambled siRNA control in combination with Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher #13778150). MMP28 knockdown was verified by qRT-PCR and western blot. Cells were transfected for 48hrs prior to in vitro experiments performed.

Quantitative Real-Time PCR

Total RNA was isolated using a RNeasy mini kit (QIAGEN). cDNA was synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystems #4368814). QRT-PCR was carried out using a TaqMan Gene Expression Master Mix (Applied Biosystems #4369016) according to manufacture protocol and TaqMan probes for human CD36 (Thermo Fisher #4331182-Hs00169627_m1), MMP28 (Thermo Fisher #4331182-Hs00425232_g1), e-cadherin/CDH1 (Thermo Fisher #4331182-Hs01023895_m1), and GAPDH (Thermo Fisher #4331182-Hs02786624_g1).

Trans-Well Invasion Assay

HCT116 CD36 Overexpressing and CD36 knockdown cell lines were plated and starved for 48hrs on 100 mm petri dishes with McCoy's 5A medium without FBS supplementation. Corning 24-well BioCoat™ Matrigel® Invasion Chambers (Corning # 354480) were rehydrated for 2hrs with McCoy's 5A medium not supplement with FBS at 37C. HCt116 cell lines were then trypsinized and counted using a Beckman Coulter Vi-Cell BLU Cell Viability Analyzer (Beckman Coulter #C19196) and plated at 50,000 cells/chamber in serum free with McCoy's 5A medium. Invasion chambers were then placed in wells containing 400 uL of McCoy's 5A medium supplemented with 10% FBS as a chemoattractant. Cell were allowed to invade for 48hrs. Chambers were then aspirated, the inside wiped with a cotton swab, and chambers stained with 0.1% crystal violet (Millipore sigma

#C6158-50G) for 10 minutes. Chambers were imaged and then de-stained using extraction buffer from the CytoSelect Cell Invasion Assay Kit (Cell Biolabs Inc. # CBA-111-T). Extracts were plated on a 96 well plate and absorbance was measured at 560 nm on a microplate reader for cell invasion quantification.

Colony Formation Assay

6-well cell culture plates were first layered with 2% soft agar using SeaPlaque™ GTG Agarose (Lonza #50115) melted in Milli-Q water. HCT116 and HT29 LuM0 CD36 overexpressing cells were tripsinized and counted as described above. Cells were mixed with a 1.5% soft agar and 2X McCoy's 5A medium solution and plated at a concentration of 5,000 cells/well. McCoy's 5A medium supplemented with 10% FBS was placed on top of both agarose layers and media levels monitored. Colonies were allowed to grow for 14 days then stained with 0.1% crystal violet (Millipore sigma #C6158-50G) for 2hrs at room temperature. Wells were then washed with Milli-Q water to remove excess crystal violet stain then imaged and colony diameter measured.

Confocal Microscopy

HT29-GFP-Luc. LuM0 and LuM3 cells were plated at a concentration of 10,00 cells/chamber onto u-Slide 8 well chambered coverslips (Ibidi #80826) in 400ul McCoy's 5A medium supplemented with 10% FBS for 48hrs. Cells were then fixed with 10% Formalin for 10 minutes followed by permeabilization with 1% Triton X. Cells were then blocked with 1% bovine serum albumin (BSA) for 20 minutes at room temperature. Cells were incubated with primary antibodies for CD36 (Santa Cruz # sc-7309), and MMP28 (Abcam #ab175937) in 1% BSA for 2hrs at room temperature. Cells were then washed 3X with PBS and incubated with fluorescent secondary antibodies (Invitrogen #A11032 and # A32728) in 1% BSA for 1hr at room temperature. Chambers were washed 3X with PBS and incubated with Hoechst and Phalloidin stains for 20 minutes. Chambers were then imaged using a Nikon A1 Confocal Microscope.

FA Uptake Assay

HT29-GFP-Luc. LuM0 and LuM3 cells were plated at 10,000 cells/well on an 8-well coverslip u-slide (Ibidi #80826) and treated with CD36 neutralizing antibody (Cayman Chemical #1009893) for 24 hrs. After incubation with neutralizing antibody, cells were then treated with fluorescent FA analogue BODIPY ™ 558/568 C_{12} (Thermo Fisher # D3835) for 10 minutes in serum free McCoy's 5A medium supplemented with 10% FA free BSA. Cells were washed twice with PBS and fixed with PBS containing 5% formalin for 20 minutes at 37C. Cell were then imaged via confocal microscopy using a Nikon A1 Confocal Microscope.

Tail-Vein Injections

HT29-LuM3-GFP-Luc. NTC (n=5) and shCD36 (n=5) cell lines were aliquoted into PBS at a concentration of 1.0x10^6 cells/100ul and injected via tail-vein into 6–8-week-old NSG mice (NOD.Cg-Prkdc Il2rg /SzJ). After 9 weeks, lung colonization was first imaged via a luciferase reporter assay and a Lago Spectral Imaging System (Lago SII). Live mice were administered 100ul of D-luciferin at a concentration of 3mg D-luciferin/20kg mouse via intraperitoneal injection. After 10 minutes, mice were imaged, and bioluminescent signal was quantified using the Lago SII. After live imaging, mice were sacrificed, and lungs resected and imaged for GFP signal. Lung tissues were processed for immunohistochemistry (IHC) as described below.

Cecum Injections

Cecum injections were performed as previously described [299]. HCT116 CD36 overexpression (n=5) and Control (n=5) cells were aliquoted in PBS at a concentration of 1.0x10^6 cells/50ul and injected into the cecal wall of NU/NU mice. After 10 weeks, mice were sacrificed and imaged for tumor burden within the cecum, colon, and intestine. Cecum tissues were collected for IHS as described below.

RNA-Seq and Gene Enrichment Analysis

RNA samples extracted from HT29-LuM0-GFP-Luc. Control and CD36 Overexpression cell lines were analyzed via RNA-Seq by BGI Genomics, Cambridge, MA. Gene enrichment analysis was performed on HT29-LuM0-GFP-Luc. Control and CD36 Overexpression cell lines by the Biostatistics and Bioinformatics Shared Resource Facility at the University of Kentucky, Lexington, KY.

APPENDIX 3. METHODS FOR CHAPTER 4

Mouse Strains

C57BL/6J mice with LoxP-flanked FASN alleles were obtained from Clay Semenkovich, MD at Washington University and FASN/VillinCre, $FASN^{+/\Delta}$ /Apc/Villin-Cre and $FASN^{\Delta/\Delta}$ /Apc/Villin-Cre mouse colonies along with their ERT2 derivatives were established in Dr. Zaytseva's laboratory.

Animal Survival Studies

Wild type and FASN heterozygous knockout mouse strains, both male and female, monitored for survival. Mice were sacrificed near experiment termination

and their life span in days was counted. Tissues were collected for imaging of tumor burden. Number of adenomas was counted within 10 cm of small intestine (starting) 1 cm from the cecum. Swiss rolls were prepared to confirm histology and perform IHC on FASN, Ki67 and other markers. qRT-PCR and western blot analysis were used to asses FASN expression.

Metabolite Extraction

Isolated tumors from mouse strains described above were removed from cryostorage and transferred to a mircovial set for use with a Freezer/Mill Cryogenic Grinder (SPEX SamplePrep model 6875D). Tissue was pulvierzied to 5um particles. Metabolites were extracted directly from the microvial by the addition of 1 ml of 50% methanol containing 20 M L-norvaline (procedural, internal control) and separated into polar (aqueous layer) and insoluble pellet (protein/DNA/RNA/glycogen) by centrifugation at 4°C, 15,000rpm for 10 minutes. The pellet was subsequently washed four times with 50% methanol and once with 100% methanol to remove polar contaminants. The pellet was then hydrolyzed in 200ul of 3N hydrochloric acid and then neutralized with 200ul of 100% methanol. The polar and pellet fraction was dried at 10-3 mBar using a SpeedVac (Thermo Fisher) followed by derivatization. The insoluble pellet was hydrolyzed similar to the technique described in [342].

Sample Derivatization and Gas Chromatography-Mass Spectrometry Quantificantion

Dried polar and insoluble samples were derivatized by the addition of 50ul 20 mg/ml methoxyamine hydrochloride in pyridine, vortexed thoroughly and incubated for 1.5 hrs at 30°C. Sequential addition of 80ul of N-methyl-trimethylsilyl-trifluoroacetamide (MSTFA) followed with an incubation time of 30 minutes at 37°C with thorough vortexing between addition of solvents. The mixture was then transferred to an amber, v-shaped glass chromatography vial and analyzed by GCMS.

An Agilent 7800B gas-chromatography coupled to a 5977B mass spectrometry (GCMS) detector was used for this study. GCMS protocols were similar to those described previously[343, 344] except a modified temperature gradient was used for GC: Initial temperature was 130°C, held for 4 minutes, rising at 6°C/minutes to 243°C, rising at 60°C/minutes to 280°C, held for 2 minutes. The electron ionization (EI) energy was set to 70 eV. Scan (m/z:50-800) and full scan mode were used for metabolomics analysis. Mass spectra were translated to relative metabolite abundance using MassHunter MS quantitative software matched to the FiehnLib metabolomics library (available through Agilent) for retention time and fragmentation pattern[344-346]. Relative abundance was corrected for recovery using the L-norvaline standard and adjusted to protein input represented by the sum of amino acids from the pellet fraction also analyzed by GCMS.

Reverse Phase Protein Array

The RPPA was performed by the Redox Metabolism Shared Resource Facility at The University of Kentucky. The raw RPPA data from mouse tissues described above was obtained in 6 dilution steps and was processed based on the following procedure. Firstly, data quality control was performed by plotting the relationship between background corrected intensity and dilution step. Proteins with low measurement quality as reflected by large variation across replicates or unreliable curve trend were excluded. Secondly, nonlinear curve fitting for the background corrected intensity vs dilution step was applied based on the "serial dilution curve" algorithm [347] to infer the concentration of each protein in the original undiluted sample. Due to the low protein concentration measurement in the 6^{th} dilution step, the nonlinear curve fitting was only based on data from the first 5 dilution steps. Thirdly, protein concentrations were log2-transformed and normalized based on the median normalization method as described in [348] and [349]. Finally, the Wilcoxon Rank Sum test was used for differential expression analysis comparing between experimental groups. Multiple comparisons adjustment was performed by the Benjamini and Hochberg method. Differentially expressed proteins were identified by false discovery rate < 0.05. Heatmap and volcano plots were generated to demonstrate the relative expression changes of proteins of interest.

RNA-Seq and Gene Enrichment Analysis

RNA samples extracted FASN/VillinCre and $FASN^{+/\Delta}$ /Apc/Villin-Cre were analyzed via RNA-Seq by BGI Genomics, Cambridge, MA. Gene enrichment analysis was performed on these same tissues by the Biostatistics and Bioinformatics Shared Resource Facility at the University of Kentucky, Lexington, KY.

Colon Cancer Established and Primary Cell Lines

The established cell line HCT116 was maintained in McCoy's 5A medium supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO) and 1% penicillin–streptomycin. HCT116 NTC, shGFPT1, and shOGT were stablishged via transfection of lentiviral particles targeting GFPT1, OGT, or non-targetd control ( ). Cells were selected with 10mg/mL puromycin. Knockdown was confirmed via quantitative real-time PCR (qRT-PCR) after cell selection and prior to performing animal experiments. Primary colon cancer patient Pt 93 and Pt 130 cultures were isolated and established from PDX tumors as previously described (1). Cells were maintained as monolayer culture in DMEM supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO) and 1% penicillin–streptomycin. Primary Pt 93
colon cancer cells were authenticated as unique human cell lines (Genetica).

Cell Proliferation Assay

Pt93 cells were plated onto 24 well plates at a concentration of 30,000 cells per well in DMEM medium with 10% FBS and 1% penicillin–streptomycin Cells were treated with varying concentrations of azaserine (Millipore Sigma #A4142-50MG) or OSMI1 (Millipore Sigma #SML1621-5MG). Cells were incubated at 37°C for 48 hrs. After incubation period, cells were trypsinized and collected individually based on well and condition of treatment. Cells were counted using a Vi-Cell XR Cell Viability Analyzer (Beckman Coulter #C19196).

Colony Formation Assay

6-well cell culture plates were first layered with 2% soft agar using SeaPlaque™ GTG Agarose (Lonza #50115) melted in Milli-Q water. Pt93 cells were tripsinized and counted as described above. Cells were mixed with a 1.5% soft agar and 2X McCoy's 5A medium solution and plated at a concentration of 5,000 cells/well. McCoy's 5A medium supplemented with 10% FBS and containing 20uM of either azaserine or OSMI-1, was placed on top of both agarose layers and media levels monitored. Colonies were allowed to grow for 14 days then stained with 0.1% crystal violet (Millipore sigma #C6158-50G) for 2hrs at room temperature. Wells were then washed with Milli-Q water to remove excess crystal violet stain then imaged and colony diameter measured.

Quantitative Real-Time PCR

Total RNA was isolated using a RNeasy mini kit (QIAGEN). cDNA was synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystems #4368814). QRT-PCR was carried out using a TaqMan Gene Expression Master Mix (Applied Biosystems #4369016) according to manufacture protocol and TaqMan probes for human FASN (Thermo Fisher #4331182-Hs01005622_m1), GFPT1 (Thermo Fisher #4331182-Hs00899865_m1), OGT (Thermo Fisher #4331182-Hs00269228_m1), and GAPDH (Thermo Fisher #4331182-Hs02786624_g1).

Subcutaneous Xenografts

NU/NU mice were injected subcutaneously with 1.0×10^6 cells of HCT116 NTC (non-targeted control, n=5), shGFPT1 (n=5) or shOGT (n=7) in 100 µL PBS and tumor growth was monitored. Tumor size was measured via calipers every three days and tumor volume was calculated using the formula: $TV = width^2 \times length / 0.52$. When NTC tumor growth reached approximately 200 mm^3, all mice were sacrificed, and tumor weight was taken via digital scale.

LIST OF REFERENCES

1. Ferlay, J., et al., *Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008.* Int J Cancer, 2010. **127**(12): p. 2893-917.
2. Siegel, R.L., et al., *Colorectal cancer statistics, 2020.* CA Cancer J Clin, 2020. **70**(3): p. 145-164.
3. Bray, F., et al., *Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries.* CA Cancer J Clin, 2018. **68**(6): p. 394-424.
4. Dekker, E., et al., *Colorectal cancer.* Lancet, 2019. **394**(10207): p. 1467-1480.
5. Rawla, P., T. Sunkara, and A. Barsouk, *Epidemiology of colorectal cancer: incidence, mortality, survival, and risk factors.* Prz Gastroenterol, 2019. **14**(2): p. 89-103.
6. Vega, P., F. Valentin, and J. Cubiella, *Colorectal cancer diagnosis: Pitfalls and opportunities.* World J Gastrointest Oncol, 2015. **7**(12): p. 422-33.
7. Garborg, K., et al., *Current status of screening for colorectal cancer.* Ann Oncol, 2013. **24**(8): p. 1963-72.
8. Edwards, B.K., et al., *Annual report to the nation on the status of cancer, 1975-2006, featuring colorectal cancer trends and impact of interventions (risk factors, screening, and treatment) to reduce future rates.* Cancer, 2010. **116**(3): p. 544-73.
9. Cotterchio, M., et al., *Colorectal screening is associated with reduced colorectal cancer risk: a case-control study within the population-based Ontario Familial Colorectal Cancer Registry.* Cancer Causes Control, 2005. **16**(7): p. 865-75.
10. Guinney, J., et al., *The consensus molecular subtypes of colorectal cancer.* Nat Med, 2015. **21**(11): p. 1350-6.
11. Muller, M.F., A.E. Ibrahim, and M.J. Arends, *Molecular pathological classification of colorectal cancer.* Virchows Arch, 2016. **469**(2): p. 125-34.
12. Budinska, E., et al., *Gene expression patterns unveil a new level of molecular heterogeneity in colorectal cancer.* J Pathol, 2013. **231**(1): p. 63-76.
13. Thanki, K., et al., *Consensus Molecular Subtypes of Colorectal Cancer and their Clinical Implications.* Int Biol Biomed J, 2017. **3**(3): p. 105-111.
14. Dienstmann, R., et al., *Consensus molecular subtypes and the evolution of precision medicine in colorectal cancer.* Nat Rev Cancer, 2017. **17**(4): p. 268.
15. De Sousa, E.M.F., et al., *Poor-prognosis colon cancer is defined by a molecularly distinct subtype and develops from serrated precursor lesions.* Nat Med, 2013. **19**(5): p. 614-8.
16. Cancer Genome Atlas, N., *Comprehensive molecular characterization of human colon and rectal cancer.* Nature, 2012. **487**(7407): p. 330-7.

17. Prashanth Rawla, T.S., Adam Barsouk, *Surveillance, Epidemiology, and End Results (SEER) Program (www.seer.cancer.gov) SEER*Stat Database: Incidence – SEER 18 Regs Research Data + Hurricane Katrina Impacted Louisiana Cases, Nov 2015 Sub (1973–2013 varying) – Linked To County Attributes – Total U.S., 1969–2014 Counties, National Cancer Institute, DCCPS, Surveillance Research Program, Surveillance Systems Branch, released April 2016, based on the November 2015 submission.* 2016.
18. Society., A.C. *Cancer Facts & Figures 2021 Atlanta, Ga*. 2021 [cited 2021 03/15]; Available from: https://www.cancer.org/cancer/colon-rectal-cancer/detection-diagnosis-staging/survival-rates.html#references.
19. Howlader N, N.A., Krapcho M, Miller D, Brest A, Yu M, Ruhl J, Tatalovich Z, Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA (eds). *SEER Cancer Statistics Review, 1975-2017, National Cancer Institute. Bethesda, MD, https://seer.cancer.gov/csr/1975_2017/, based on November 2019 SEER data submission, posted to the SEER web site*. 2020.
20. Lawler, M., et al., *Critical research gaps and recommendations to inform research prioritisation for more effective prevention and improved outcomes in colorectal cancer.* Gut, 2018. **67**(1): p. 179-193.
21. Lawler M, J.B., Van Schaeybroeck S, Salto-Tellez M, Wilson R, Dunlop M, and Johnston, *Abeloff's Clinical Oncology Chapter 74 – Colorectal Cancer.* 6th ed. 2020, Philadelphia, Pa: Elsevier.
22. Sargent, D., et al., *Evidence for cure by adjuvant therapy in colon cancer: observations based on individual patient data from 20,898 patients on 18 randomized trials.* J Clin Oncol, 2009. **27**(6): p. 872-7.
23. Vertessy, B.G. and J. Toth, *Keeping uracil out of DNA: physiological role, structure and catalytic mechanism of dUTPases.* Acc Chem Res, 2009. **42**(1): p. 97-106.
24. Vodenkova, S., et al., *5-fluorouracil and other fluoropyrimidines in colorectal cancer: Past, present and future.* Pharmacol Ther, 2020. **206**: p. 107447.
25. Longley, D.B., D.P. Harkin, and P.G. Johnston, *5-fluorouracil: mechanisms of action and clinical strategies.* Nat Rev Cancer, 2003. **3**(5): p. 330-8.
26. Carter, S.K., *Editorial: Large-bowel cancer-The current status of treatment.* J Natl Cancer Inst, 1976. **56**(1): p. 3-10.
27. Johnston, P.G. and S. Kaye, *Capecitabine: a novel agent for the treatment of solid tumors.* Anticancer Drugs, 2001. **12**(8): p. 639-46.
28. Laufman, L.R., et al., *Leucovorin plus 5-fluorouracil: an effective treatment for metastatic colon cancer.* J Clin Oncol, 1987. **5**(9): p. 1394-400.
29. Poon, M.A., et al., *Biochemical modulation of fluorouracil: evidence of significant improvement of survival and quality of life in patients with advanced colorectal carcinoma.* J Clin Oncol, 1989. **7**(10): p. 1407-18.
30. Comella, P., et al., *Role of oxaliplatin in the treatment of colorectal cancer.* Ther Clin Risk Manag, 2009. **5**(1): p. 229-38.
31. Rosenberg, B., et al., *Platinum compounds: a new class of potent antitumour agents.* Nature, 1969. **222**(5191): p. 385-6.

32. de Gramont, A., et al., *Leucovorin and fluorouracil with or without oxaliplatin as first-line treatment in advanced colorectal cancer.* J Clin Oncol, 2000. **18**(16): p. 2938-47.
33. Andre, T., et al., *Bimonthly high-dose leucovorin, 5-fluorouracil infusion and oxaliplatin (FOLFOX3) for metastatic colorectal cancer resistant to the same leucovorin and 5-fluorouracil regimen.* Ann Oncol, 1998. **9**(11): p. 1251-3.
34. de Gramont, A., et al., *Oxaliplatin with high-dose leucovorin and 5-fluorouracil 48-hour continuous infusion in pretreated metastatic colorectal cancer.* Eur J Cancer, 1997. **33**(2): p. 214-9.
35. Xie, Y.H., Y.X. Chen, and J.Y. Fang, *Comprehensive review of targeted therapy for colorectal cancer.* Signal Transduct Target Ther, 2020. **5**(1): p. 22.
36. Roskoski, R., Jr., *The ErbB/HER family of protein-tyrosine kinases and cancer.* Pharmacol Res, 2014. **79**: p. 34-74.
37. Takegawa, N. and K. Yonesaka, *HER2 as an Emerging Oncotarget for Colorectal Cancer Treatment After Failure of Anti-Epidermal Growth Factor Receptor Therapy.* Clin Colorectal Cancer, 2017. **16**(4): p. 247-251.
38. Wang, Z., *ErbB Receptors and Cancer.* Methods Mol Biol, 2017. **1652**: p. 3-35.
39. Mendelsohn, J., et al., *CCR 20th anniversary commentary: a chimeric antibody, C225, inhibits EGFR activation and tumor growth.* Clin Cancer Res, 2015. **21**(2): p. 227-9.
40. Yarom, N. and D.J. Jonker, *The role of the epidermal growth factor receptor in the mechanism and treatment of colorectal cancer.* Discov Med, 2011. **11**(57): p. 95-105.
41. Clarke, C.N. and E.S. Kopetz, *BRAF mutant colorectal cancer as a distinct subset of colorectal cancer: clinical characteristics, clinical behavior, and response to targeted therapies.* J Gastrointest Oncol, 2015. **6**(6): p. 660-7.
42. Corcoran, R.B., et al., *Combined BRAF, EGFR, and MEK Inhibition in Patients with BRAF(V600E)-Mutant Colorectal Cancer.* Cancer Discov, 2018. **8**(4): p. 428-443.
43. Corcoran, R.B., et al., *EGFR-mediated re-activation of MAPK signaling contributes to insensitivity of BRAF mutant colorectal cancers to RAF inhibition with vemurafenib.* Cancer Discov, 2012. **2**(3): p. 227-35.
44. Lito, P., et al., *Relief of profound feedback inhibition of mitogenic signaling by RAF inhibitors attenuates their activity in BRAFV600E melanomas.* Cancer Cell, 2012. **22**(5): p. 668-82.
45. Yonesaka, K., et al., *Activation of ERBB2 signaling causes resistance to the EGFR-directed therapeutic antibody cetuximab.* Sci Transl Med, 2011. **3**(99): p. 99ra86.
46. Meric-Bernstam, F., et al., *Pertuzumab plus trastuzumab for HER2-amplified metastatic colorectal cancer (MyPathway): an updated report from a multicentre, open-label, phase 2a, multiple basket study.* Lancet Oncol, 2019. **20**(4): p. 518-530.
47. Sartore-Bianchi, A., et al., *Dual-targeted therapy with trastuzumab and lapatinib in treatment-refractory, KRAS codon 12/13 wild-type, HER2-positive metastatic colorectal cancer (HERACLES): a proof-of-concept, multicentre, open-label, phase 2 trial.* Lancet Oncol, 2016. **17**(6): p. 738-746.

48. Folkman, J., *Tumor angiogenesis: therapeutic implications.* N Engl J Med, 1971. **285**(21): p. 1182-6.
49. Vlajnic, T., et al., *VEGFA gene locus (6p12) amplification identifies a small but highly aggressive subgroup of colorectal cancer [corrected] patients.* Mod Pathol, 2011. **24**(10): p. 1404-12.
50. Hurwitz, H., et al., *Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer.* N Engl J Med, 2004. **350**(23): p. 2335-42.
51. Kienast, Y., et al., *Ang-2-VEGF-A CrossMab, a novel bispecific human IgG1 antibody blocking VEGF-A and Ang-2 functions simultaneously, mediates potent antitumor, antiangiogenic, and antimetastatic efficacy.* Clin Cancer Res, 2013. **19**(24): p. 6730-40.
52. Van Cutsem, E., et al., *Nintedanib for the treatment of patients with refractory metastatic colorectal cancer (LUME-Colon 1): a phase III, international, randomized, placebo-controlled study.* Ann Oncol, 2018. **29**(9): p. 1955-1963.
53. Semrad, T.J., et al., *Phase II Study of Dovitinib in Patients Progressing on Anti-Vascular Endothelial Growth Factor Therapy.* Cancer Treat Res Commun, 2017. **10**: p. 21-26.
54. Fritz, J.M. and M.J. Lenardo, *Development of immune checkpoint therapy for cancer.* J Exp Med, 2019. **216**(6): p. 1244-1254.
55. Freeman, G.J., et al., *Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation.* J Exp Med, 2000. **192**(7): p. 1027-34.
56. Wang, C., et al., *In vitro characterization of the anti-PD-1 antibody nivolumab, BMS-936558, and in vivo toxicology in non-human primates.* Cancer Immunol Res, 2014. **2**(9): p. 846-56.
57. Xu-Monette, Z.Y., et al., *PD-1/PD-L1 Blockade: Have We Found the Key to Unleash the Antitumor Immune Response?* Frontiers in Immunology, 2017. **8**(1597).
58. Le, D.T., et al., *PD-1 Blockade in Tumors with Mismatch-Repair Deficiency.* N Engl J Med, 2015. **372**(26): p. 2509-20.
59. Overman, M.J., et al., *Nivolumab in patients with metastatic DNA mismatch repair-deficient or microsatellite instability-high colorectal cancer (CheckMate 142): an open-label, multicentre, phase 2 study.* Lancet Oncol, 2017. **18**(9): p. 1182-1191.
60. Winawer, S.J., et al., *Prevention of colorectal cancer by colonoscopic polypectomy. The National Polyp Study Workgroup.* N Engl J Med, 1993. **329**(27): p. 1977-81.
61. Smith, R.A., et al., *Cancer screening in the United States, 2010: a review of current American Cancer Society guidelines and issues in cancer screening.* CA Cancer J Clin, 2010. **60**(2): p. 99-119.
62. *Colorectal (Colon) Cancer, Use of Colorectal Cancer Screening Tests, Division of Cancer Prevention and Control*. 2021 [cited 2021 05/24/2021].
63. Siegel, R.L., et al., *Colorectal Cancer Incidence Patterns in the United States, 1974-2013.* J Natl Cancer Inst, 2017. **109**(8).

64. Riihimaki, M., et al., *Patterns of metastasis in colon and rectal cancer.* Sci Rep, 2016. **6**: p. 29765.
65. Haggar, F.A. and R.P. Boushey, *Colorectal cancer epidemiology: incidence, mortality, survival, and risk factors.* Clin Colon Rectal Surg, 2009. **22**(4): p. 191-7.
66. Maas, M., et al., *Long-term outcome in patients with a pathological complete response after chemoradiation for rectal cancer: a pooled analysis of individual patient data.* Lancet Oncol, 2010. **11**(9): p. 835-44.
67. Walker, A.S., et al., *Future directions for monitoring treatment response in colorectal cancer.* J Cancer, 2014. **5**(1): p. 44-57.
68. de Campos-Lobato, L.F., et al., *Pathologic complete response after neoadjuvant treatment for rectal cancer decreases distant recurrence and could eradicate local recurrence.* Ann Surg Oncol, 2011. **18**(6): p. 1590-8.
69. Rosen, S.A., et al., *Initial Presentation With Stage IV Colorectal Cancer: How Aggressive Should We Be?* Archives of Surgery, 2000. **135**(5): p. 530-534.
70. Jeffery, M., et al., *Follow-up strategies for patients treated for non-metastatic colorectal cancer.* Cochrane Database of Systematic Reviews, 2016(11).
71. Duineveld, L.A., et al., *Symptomatic and Asymptomatic Colon Cancer Recurrence: A Multicenter Cohort Study.* Ann Fam Med, 2016. **14**(3): p. 215-20.
72. Bohm, B., et al., *Does methodic long-term follow-up affect survival after curative resection of colorectal carcinoma?* Dis Colon Rectum, 1993. **36**(3): p. 280-6.
73. Fleischer, D.E., et al., *Detection and surveillance of colorectal cancer.* JAMA, 1989. **261**(4): p. 580-5.
74. Sugarbaker, P.H., et al., *A simplified plan for follow-up of patients with colon and rectal cancer supported by prospective studies of laboratory and radiologic test results.* Surgery, 1987. **102**(1): p. 79-87.
75. Alberts, S.R., et al., *Oxaliplatin, fluorouracil, and leucovorin for patients with unresectable liver-only metastases from colorectal cancer: a North Central Cancer Treatment Group phase II study.* J Clin Oncol, 2005. **23**(36): p. 9243-9.
76. Muratore, A., et al., *Asymptomatic colorectal cancer with un-resectable liver metastases: immediate colorectal resection or up-front systemic chemotherapy?* Ann Surg Oncol, 2007. **14**(2): p. 766-70.
77. Choti, M.A., et al., *Trends in long-term survival following liver resection for hepatic colorectal metastases.* Ann Surg, 2002. **235**(6): p. 759-66.
78. Pakiet, A., et al., *Changes in lipids composition and metabolism in colorectal cancer: a review.* Lipids in Health and Disease, 2019. **18**(1).
79. Gunstone, F.D., *Fatty Acid and Lipid Chemistry*. 1st ed. Fatty Acids - Nomenclature, structure, isolation and structure determination, biosynthesis and chemical synthesis. 1996, United Kingdom: Springer Science.
80. Turner, N., et al., *Fatty acid metabolism, energy expenditure and insulin resistance in muscle.* Journal of Endocrinology, 2014. **220**(2): p. T61-T79.
81. Powell, L. and E.C. Wallace, *79 - Fatty Acid Metabolism*, in *Textbook of Natural Medicine (Fifth Edition)*, J.E. Pizzorno and M.T. Murray, Editors. 2020, Churchill Livingstone: St. Louis (MO). p. 584-592.e4.

82. Christie, W.W. *Sterols: 1. Cholesterol and Cholesterol Esters*. 2021 May 19th, 2021 [cited 2021 5/25/21].
83. Britannica, T.E.o.E. *Phospholipid*. 2009 [cited 2021 06/01/2021]; Available from: https://www.britannica.com/science/phospholipid.
84. Wang, T.Y., et al., *New insights into the molecular mechanism of intestinal fatty acid absorption.* Eur J Clin Invest, 2013. **43**(11): p. 1203-23.
85. GM, C. *The Cell: A Molecular Approach. 2nd edition*. 2000; Available from: https://www.ncbi.nlm.nih.gov/books/NBK9928/.
86. van Meer, G., D.R. Voelker, and G.W. Feigenson, *Membrane lipids: where they are and how they behave.* Nat Rev Mol Cell Biol, 2008. **9**(2): p. 112-24.
87. Alberts B, J.A., Lewis J, et al, *Molecular Biology of the Cell*. 4th ed. The Lipid Bilayer. 2002, New York: Garland Science.
88. Gennis, R.B., *Biomembranes : molecular structure and function*. Springer advanced texts in chemistry. 1989, New York: Springer-Verlag. xvii, 533 p.
89. de Carvalho, C. and M.J. Caramujo, *The Various Roles of Fatty Acids.* Molecules, 2018. **23**(10).
90. Zhang, J., et al., *Cholesterol content in cell membrane maintains surface levels of ErbB2 and confers a therapeutic vulnerability in ErbB2-positive breast cancer.* Cell Commun Signal, 2019. **17**(1): p. 15.
91. Joseph and Michael, *A Century of Cholesterol and Coronaries: From Plaques to Genes to Statins.* Cell, 2015. **161**(1): p. 161-172.
92. Chapman, D., *Phase transitions and fluidity characteristics of lipids and cell membranes.* Q Rev Biophys, 1975. **8**(2): p. 185-235.
93. Jaipuria, G., T. Ukmar-Godec, and M. Zweckstetter, *Challenges and approaches to understand cholesterol-binding impact on membrane protein function: an NMR view.* Cell Mol Life Sci, 2018. **75**(12): p. 2137-2151.
94. Merrill, A.H., *CHAPTER 13 - Sphingolipids*, in *Biochemistry of Lipids, Lipoproteins and Membranes (Fifth Edition)*, D.E. Vance and J.E. Vance, Editors. 2008, Elsevier: San Diego. p. 363-397.
95. Ohanian, J. and V. Ohanian, *Sphingolipids in mammalian cell signalling.* Cell Mol Life Sci, 2001. **58**(14): p. 2053-68.
96. Sankaram, M.B. and T.E. Thompson, *Interaction of cholesterol with various glycerophospholipids and sphingomyelin.* Biochemistry, 1990. **29**(47): p. 10670-5.
97. Korade, Z. and A.K. Kenworthy, *Lipid rafts, cholesterol, and the brain.* Neuropharmacology, 2008. **55**(8): p. 1265-1273.
98. Grimm, M.O.W., et al., *Altered membrane fluidity and lipid raft composition in presenilin-deficient cells.* Acta Neurologica Scandinavica, 2006. **114**(s185): p. 27-32.
99. Simons, K. and D. Toomre, *Lipid rafts and signal transduction.* Nature Reviews Molecular Cell Biology, 2000. **1**(1): p. 31-39.
100. Nathan, C., *Points of control in inflammation.* Nature, 2002. **420**(6917): p. 846-52.
101. Ricciotti, E. and G.A. FitzGerald, *Prostaglandins and inflammation.* Arterioscler Thromb Vasc Biol, 2011. **31**(5): p. 986-1000.

102. Neitzel, J.J., *Fatty Acid Molecules: A Role in Cell Signaling.* Nature Education, 2010.
103. Hannun, Y.A., *Functions of ceramide in coordinating cellular responses to stress.* Science, 1996. **274**: p. 1855+.
104. Basu, S. and R. Kolesnick, *Stress signals for apoptosis: ceramide and c-Jun kinase.* Oncogene, 1998. **17**(25): p. 3277-85.
105. Alessenko, A.V., *The role of sphingomyelin cycle metabolites in transduction of signals of cell proliferation, differentiation and death.* Membr Cell Biol, 2000. **13**(2): p. 303-20.
106. Maceyka, M., et al., *Sphingosine-1-phosphate signaling and its role in disease.* Trends Cell Biol, 2012. **22**(1): p. 50-60.
107. Olivera, A. and S. Spiegel, *Sphingosine-1-phosphate as second messenger in cell proliferation induced by PDGF and FCS mitogens.* Nature, 1993. **365**(6446): p. 557-60.
108. Cuvillier, O., et al., *Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate.* Nature, 1996. **381**(6585): p. 800-3.
109. Papackova, Z. and M. Cahova, *Fatty acid signaling: the new function of intracellular lipases.* Int J Mol Sci, 2015. **16**(2): p. 3831-55.
110. Eberle, D., et al., *SREBP transcription factors: master regulators of lipid homeostasis.* Biochimie, 2004. **86**(11): p. 839-48.
111. Jackson, K.G., et al., *Saturated fat-induced changes in Sf 60-400 particle composition reduces uptake of LDL by HepG2 cells.* J Lipid Res, 2006. **47**(2): p. 393-403.
112. Jump, D.B., et al., *Fatty acid regulation of hepatic gene transcription.* J Nutr, 2005. **135**(11): p. 2503-6.
113. Stillwell, W., *An Introduction to Biological Membranes : Composition, Structure and Function*. 2016, Oxford, NETHERLANDS, THE: Elsevier Science & Technology.
114. Calder, P.C., *Fatty Acids: Metabolism*, in *Encyclopedia of Food and Health*, B. Caballero, P.M. Finglas, and F. Toldrá, Editors. 2016, Academic Press: Oxford. p. 632-644.
115. Lopaschuk, G.D., et al., *Myocardial fatty acid metabolism in health and disease.* Physiol Rev, 2010. **90**(1): p. 207-58.
116. Wakil, S.J. and L.A. Abu-Elheiga, *Fatty acid metabolism: target for metabolic syndrome.* Journal of Lipid Research, 2009. **50**: p. S138-S143.
117. Watkins, P.A., *Fatty acid activation.* Progress in Lipid Research, 1997. **36**(1): p. 55-83.
118. Dunn J, G.M. *Physiology, Adenosine Triphosphate.* StatPearls [Internet] 02/27/2021 [cited 2021 06/02]; Available from: https://www.ncbi.nlm.nih.gov/books/NBK553175/.
119. van der Vusse, G.J., *Albumin as Fatty Acid Transporter.* Drug Metabolism and Pharmacokinetics, 2009. **24**(4): p. 300-307.
120. Schwenk, R.W., et al., *Fatty acid transport across the cell membrane: regulation by fatty acid transporters.* Prostaglandins Leukot Essent Fatty Acids, 2010. **82**(4-6): p. 149-54.

121. Chakravarty, B., et al., *Human fatty acid synthase: Structure and substrate selectivity of the thioesterase domain.* Proceedings of the National Academy of Sciences, 2004. **101**(44): p. 15567-15572.
122. Koundouros, N. and G. Poulogiannis, *Reprogramming of fatty acid metabolism in cancer.* British Journal of Cancer, 2020. **122**(1): p. 4-22.
123. Cheng, C., et al., *Lipid metabolism reprogramming and its potential targets in cancer.* Cancer Communications, 2018. **38**(1): p. 27.
124. Goldstein, J.L., R.A. DeBose-Boyd, and M.S. Brown, *Protein sensors for membrane sterols.* Cell, 2006. **124**(1): p. 35-46.
125. Catalina-Rodriguez, O., et al., *The mitochondrial citrate transporter, CIC, is essential for mitochondrial homeostasis.* Oncotarget, 2012. **3**(10): p. 1220-1235.
126. Convertini, P., et al., *The contribution of the citrate pathway to oxidative stress in Down syndrome.* Immunology, 2016. **149**(4): p. 423-431.
127. Martin, G., et al., *Coordinate regulation of the expression of the fatty acid transport protein and acyl-CoA synthetase genes by PPARalpha and PPARgamma activators.* J Biol Chem, 1997. **272**(45): p. 28210-7.
128. Pavlova, N.N. and C.B. Thompson, *The Emerging Hallmarks of Cancer Metabolism.* Cell Metab, 2016. **23**(1): p. 27-47.
129. Buckley, D., et al., *Fatty acid synthase - Modern tumor cell biology insights into a classical oncology target.* Pharmacol Ther, 2017. **177**: p. 23-31.
130. Currie, E., et al., *Cellular fatty acid metabolism and cancer.* Cell Metab, 2013. **18**(2): p. 153-61.
131. Mashima, T., H. Seimiya, and T. Tsuruo, *De novo fatty-acid synthesis and related pathways as molecular targets for cancer therapy.* British Journal of Cancer, 2009. **100**(9): p. 1369-1372.
132. Abramson, H.N., *The Lipogenesis Pathway as a Cancer Target.* Journal of Medicinal Chemistry, 2011. **54**(16): p. 5615-5638.
133. Granchi, C., *ATP citrate lyase (ACLY) inhibitors: An anti-cancer strategy at the crossroads of glucose and lipid metabolism.* European Journal of Medicinal Chemistry, 2018. **157**: p. 1276-1291.
134. Csanadi, A., et al., *Prognostic Value of Malic Enzyme and ATP-Citrate Lyase in Non-Small Cell Lung Cancer of the Young and the Elderly.* PLOS ONE, 2015. **10**(5): p. e0126357.
135. Hatzivassiliou, G., et al., *ATP citrate lyase inhibition can suppress tumor cell growth.* Cancer Cell, 2005. **8**(4): p. 311-321.
136. Yahagi, N., et al., *Co-ordinate activation of lipogenic enzymes in hepatocellular carcinoma.* European Journal of Cancer, 2005. **41**(9): p. 1316-1322.
137. Turyn, J., et al., *Increased activity of glycerol 3-phosphate dehydrogenase and other lipogenic enzymes in human bladder cancer.* Horm Metab Res, 2003. **35**(10): p. 565-9.
138. Wang, D., et al., *ATP citrate lyase is increased in human breast cancer, depletion of which promotes apoptosis.* Tumor Biology, 2017. **39**(4): p. 101042831769833.

139. Bertilsson, H., et al., *Changes in Gene Transcription Underlying the Aberrant Citrate and Choline Metabolism in Human Prostate Cancer Samples.* Clinical Cancer Research, 2012. **18**(12): p. 3261-3269.
140. Guo, D., et al., *An LXR Agonist Promotes Glioblastoma Cell Death through Inhibition of an EGFR/AKT/SREBP-1/LDLR–Dependent Pathway.* Cancer Discovery, 2011. **1**(5): p. 442-456.
141. Guaita-Esteruelas, S., et al., *Exogenous FABP4 increases breast cancer cell proliferation and activates the expression of fatty acid transport proteins.* Molecular Carcinogenesis, 2017. **56**(1): p. 208-217.
142. Kim, Y.-S., et al., *High Membranous Expression of Fatty Acid Transport Protein 4 Is Associated with Tumorigenesis and Tumor Progression in Clear Cell Renal Cell Carcinoma.* Disease Markers, 2019. **2019**: p. 5702026.
143. Wen, Y.-A., et al., *Downregulation of SREBP inhibits tumor growth and initiation by altering cellular metabolism in colon cancer.* Cell Death & Disease, 2018. **9**(3): p. 265.
144. Li, N., et al., *Inhibition of the sterol regulatory element-binding protein pathway suppresses hepatocellular carcinoma by repressing inflammation in mice.* Hepatology, 2017. **65**(6): p. 1936-1947.
145. Von Roemeling, C.A., et al., *Stearoyl-CoA Desaturase 1 Is a Novel Molecular Therapeutic Target for Clear Cell Renal Cell Carcinoma.* Clinical Cancer Research, 2013. **19**(9): p. 2368-2380.
146. Li, X., et al., *Fatostatin Displays High Antitumor Activity in Prostate Cancer by Blocking SREBP-Regulated Metabolic Pathways and Androgen Receptor Signaling.* Molecular Cancer Therapeutics, 2014. **13**(4): p. 855-866.
147. Pelletier, L., et al., *Loss of hepatocyte nuclear factor 1α function in human hepatocellular adenomas leads to aberrant activation of signaling pathways involved in tumorigenesis.* Hepatology, 2010. **51**(2): p. 557-566.
148. Ku, C.-Y., et al., *Liver fatty acid-binding protein (L-FABP) promotes cellular angiogenesis and migration in hepatocellular carcinoma.* Oncotarget, 2016. **7**(14): p. 18229-18246.
149. Hale, J.S., et al., *Cancer stem cell-specific scavenger receptor CD36 drives glioblastoma progression.* Stem Cells, 2014. **32**(7): p. 1746-58.
150. Pascual, G., et al., *Targeting metastasis-initiating cells through the fatty acid receptor CD36.* Nature, 2017. **541**(7635): p. 41-45.
151. Ladanyi, A., et al., *Adipocyte-induced CD36 expression drives ovarian cancer progression and metastasis.* Oncogene, 2018. **37**(17): p. 2285-2301.
152. Watt, M.J., et al., *Suppressing fatty acid uptake has therapeutic effects in preclinical models of prostate cancer.* Science Translational Medicine, 2019. **11**(478): p. eaau5758.
153. Zaytseva, Y.Y., et al., *Inhibition of fatty acid synthase attenuates CD44-associated signaling and reduces metastasis in colorectal cancer.* Cancer Res, 2012. **72**(6): p. 1504-17.

154. Zaytseva, Y.Y., et al., *Increased expression of fatty acid synthase provides a survival advantage to colorectal cancer cells via upregulation of cellular respiration.* Oncotarget, 2015. **6**(22): p. 18891-904.
155. Chirala, S.S. and S.J. Wakil, *Structure and function of animal fatty acid synthase.* Lipids, 2004. **39**(11): p. 1045-1053.
156. Chirala, S.S., et al., *Human fatty acid synthase: role of interdomain in the formation of catalytically active synthase dimer.* Proc Natl Acad Sci U S A, 2001. **98**(6): p. 3104-8.
157. Wakil, S.J., E.L. Pugh, and F. Sauer, *The Mechanism of Fatty Acid Synthesis.* Proc Natl Acad Sci U S A, 1964. **52**: p. 106-14.
158. Gibson, D.M., E.B. Titchener, and S.J. Wakil, *Studies on the mechanism of fatty acid synthesis. V. Bicarbonate requirement for the synthesis of long-chain fatty acids.* Biochim Biophys Acta, 1958. **30**(2): p. 376-83.
159. Zhao, W., *Fatty Acid Synthase*. Springer Berlin Heidelberg. p. 1097-1099.
160. Sul, H.S., et al., *Regulation of the Fatty Acid Synthase Promoter by Insulin.* The Journal of Nutrition, 2000. **130**(2): p. 315S-320S.
161. Latasa, M.J., et al., *Nutritional regulation of the fatty acid synthase promoter in vivo: sterol regulatory element binding protein functions through an upstream region containing a sterol regulatory element.* Proc Natl Acad Sci U S A, 2000. **97**(19): p. 10619-24.
162. Paulauskis, J.D. and H.S. Sul, *Hormonal regulation of mouse fatty acid synthase gene transcription in liver.* J Biol Chem, 1989. **264**(1): p. 574-7.
163. Griffin, M.J., et al., *Direct Interaction between USF and SREBP-1c Mediates Synergistic Activation of the Fatty-acid Synthase Promoter.* Journal of Biological Chemistry, 2007. **282**(8): p. 5453-5467.
164. Shimano, H., *Sterol regulatory element-binding proteins (SREBPs): transcriptional regulators of lipid synthetic genes.* Prog Lipid Res, 2001. **40**(6): p. 439-52.
165. Latasa, M.J., et al., *Occupancy and function of the -150 sterol regulatory element and -65 E-box in nutritional regulation of the fatty acid synthase gene in living animals.* Mol Cell Biol, 2003. **23**(16): p. 5896-907.
166. Moon, Y.S., et al., *Two 5'-regions are required for nutritional and insulin regulation of the fatty-acid synthase promoter in transgenic mice.* J Biol Chem, 2000. **275**(14): p. 10121-7.
167. Ericsson, J., et al., *Sterol regulatory element binding protein binds to a cis element in the promoter of the farnesyl diphosphate synthase gene.* Proc Natl Acad Sci U S A, 1996. **93**(2): p. 945-50.
168. Wakil, S.J., J.K. Stoops, and V.C. Joshi, *Fatty acid synthesis and its regulation.* Annu Rev Biochem, 1983. **52**: p. 537-79.
169. Wakil, S.J., *Fatty acid synthase, a proficient multifunctional enzyme.* Biochemistry, 1989. **28**(11): p. 4523-30.
170. Chirala, S.S., et al., *Fatty acid synthesis is essential in embryonic development: Fatty acid synthase null mutants and most of the heterozygotes die in utero.* Proceedings of the National Academy of Sciences, 2003. **100**(11): p. 6358-6363.

171. Hannun, Y.A. and L.M. Obeid, *The Ceramide-centric universe of lipid-mediated cell regulation: stress encounters of the lipid kind.* J Biol Chem, 2002. **277**(29): p. 25847-50.
172. Lu, T., et al., *Fatty acid synthase enhances colorectal cancer cell proliferation and metastasis via regulating AMPK/mTOR pathway.* Onco Targets Ther, 2019. **12**: p. 3339-3347.
173. Orian-Rousseau, V., *CD44, a therapeutic target for metastasising tumours.* Eur J Cancer, 2010. **46**(7): p. 1271-7.
174. Pizer, E.S., et al., *Inhibition of fatty acid synthesis induces programmed cell death in human breast cancer cells.* Cancer Res, 1996. **56**(12): p. 2745-7.
175. Pizer, E.S., et al., *Inhibition of fatty acid synthesis delays disease progression in a xenograft model of ovarian cancer.* Cancer Res, 1996. **56**(6): p. 1189-93.
176. Rendina, A.R. and D. Cheng, *Characterization of the inactivation of rat fatty acid synthase by C75: inhibition of partial reactions and protection by substrates.* Biochem J, 2005. **388**(Pt 3): p. 895-903.
177. Flavin, R., et al., *Fatty acid synthase as a potential therapeutic target in cancer.* Future Oncol, 2010. **6**(4): p. 551-62.
178. Funabashi, H., et al., *Binding site of cerulenin in fatty acid synthetase.* J Biochem, 1989. **105**(5): p. 751-5.
179. Loftus, T.M., et al., *Reduced food intake and body weight in mice treated with fatty acid synthase inhibitors.* Science, 2000. **288**(5475): p. 2379-81.
180. Kridel, S.J., et al., *Orlistat is a novel inhibitor of fatty acid synthase with antitumor activity.* Cancer Res, 2004. **64**(6): p. 2070-5.
181. Hoover, H.S., et al., *Selectivity of inhibitors of endocannabinoid biosynthesis evaluated by activity-based protein profiling.* Bioorg Med Chem Lett, 2008. **18**(22): p. 5838-41.
182. Evanchik, M., et al., *TVB-2640, a Novel Anti-HCV Agent, Safely Causes Sustained Host-Target Inhibition in Vivo: 1876.* Hepatology, 2012. **56**.
183. Oslob, J.D., et al., *Imidazopyridine-Based Fatty Acid Synthase Inhibitors That Show Anti-HCV Activity and in Vivo Target Modulation.* ACS Med Chem Lett, 2013. **4**(1): p. 113-7.
184. Ventura, R., et al., *Inhibition of de novo Palmitate Synthesis by Fatty Acid Synthase Induces Apoptosis in Tumor Cells by Remodeling Cell Membranes, Inhibiting Signaling Pathways, and Reprogramming Gene Expression.* EBioMedicine, 2015. **2**(8): p. 808-824.
185. Zaytseva, Y.Y., et al., *Preclinical evaluation of novel fatty acid synthase inhibitors in primary colorectal cancer cells and a patient-derived xenograft model of colorectal cancer.* Oncotarget, 2018. **9**(37): p. 24787-24800.
186. Aquino, I.G.d., et al., *Anticancer properties of the fatty acid synthase inhibitor TVB-3166 on oral squamous cell carcinoma cell lines.* Archives of Oral Biology, 2020. **113**: p. 104707.
187. Herter-Sprie, G.S., A.L. Kung, and K.K. Wong, *New cast for a new era: preclinical cancer drug development revisited.* J Clin Invest, 2013. **123**(9): p. 3639-45.

188. Siolas, D. and G.J. Hannon, *Patient-derived tumor xenografts: transforming clinical samples into mouse models.* Cancer Res, 2013. **73**(17): p. 5315-9.
189. Seol, H.S., et al., *Development and characterization of a colon PDX model that reproduces drug responsiveness and the mutation profiles of its original tumor.* Cancer Lett, 2014. **345**(1): p. 56-64.
190. Aparicio, S., M. Hidalgo, and A.L. Kung, *Examining the utility of patient-derived xenograft mouse models.* Nat Rev Cancer, 2015. **15**(5): p. 311-6.
191. *FASN Inhibitor TVB-2640, Paclitaxel, and Trastuzumab in Treating Patients With HER2 Positive Advanced Breast Cancer*. 2017 [cited 2021 06/04/2021]; Available from: https://clinicaltrials.gov/ct2/show/NCT03179904.
192. *TVB 2640 for Resectable Colon Cancer Other Resectable Cancers; a Window Trial.* 2016 [cited 2021 06/04/2021]; Available from: https://clinicaltrials.gov/ct2/show/NCT02980029.
193. *Phase 2 Study of TVB-2640 in KRAS Non-Small Cell Lung Carcinomas*. 2019 [cited 2021 06/04/2021]; Available from: https://clinicaltrials.gov/ct2/show/NCT03808558.
194. Pepino, M.Y., et al., *Structure-function of CD36 and importance of fatty acid signal transduction in fat metabolism.* Annu Rev Nutr, 2014. **34**: p. 281-303.
195. Canton, J., D. Neculai, and S. Grinstein, *Scavenger receptors in homeostasis and immunity.* Nat Rev Immunol, 2013. **13**(9): p. 621-34.
196. Kelley, L.A. and M.J. Sternberg, *Protein structure prediction on the Web: a case study using the Phyre server.* Nat Protoc, 2009. **4**(3): p. 363-71.
197. Neculai, D., et al., *Structure of LIMP-2 provides functional insights with implications for SR-BI and CD36.* Nature, 2013. **504**(7478): p. 172-6.
198. Kuda, O., et al., *Sulfo-N-succinimidyl oleate (SSO) inhibits fatty acid uptake and signaling for intracellular calcium via binding CD36 lysine 164: SSO also inhibits oxidized low density lipoprotein uptake by macrophages.* J Biol Chem, 2013. **288**(22): p. 15547-55.
199. Storch, J. and L. McDermott, *Structural and functional analysis of fatty acid-binding proteins.* J Lipid Res, 2009. **50 Suppl**: p. S126-31.
200. Flores, J.J., et al., *PPARgamma-induced upregulation of CD36 enhances hematoma resolution and attenuates long-term neurological deficits after germinal matrix hemorrhage in neonatal rats.* Neurobiol Dis, 2016. **87**: p. 124-33.
201. Tontonoz, P., et al., *PPARgamma promotes monocyte/macrophage differentiation and uptake of oxidized LDL.* Cell, 1998. **93**(2): p. 241-52.
202. Sp, N., et al., *Nobiletin Inhibits CD36-Dependent Tumor Angiogenesis, Migration, Invasion, and Sphere Formation Through the Cd36/Stat3/Nf-Kappab Signaling Axis.* Nutrients, 2018. **10**(6).
203. Lehner, R. and A.D. Quiroga, *Fatty Acid Handling in Mammalian Cells*. 2016, Elsevier. p. 149-184.
204. Hoosdally, S.J., et al., *The Human Scavenger Receptor CD36.* Journal of Biological Chemistry, 2009. **284**(24): p. 16277-16288.
205. Su, X. and N.A. Abumrad, *Cellular fatty acid uptake: a pathway under construction.* Trends Endocrinol Metab, 2009. **20**(2): p. 72-7.

206. *CD36*. Tissue Atlas 2021 [cited 2021 06/09/2021]; Available from: https://www.proteinatlas.org/ENSG00000135218-CD36/tissue.
207. Silverstein, R.L., et al., *Sense and antisense cDNA transfection of CD36 (glycoprotein IV) in melanoma cells. Role of CD36 as a thrombospondin receptor.* J Biol Chem, 1992. **267**(23): p. 16607-12.
208. Osz, K., M. Ross, and J. Petrik, *The thrombospondin-1 receptor CD36 is an important mediator of ovarian angiogenesis and folliculogenesis.* Reprod Biol Endocrinol, 2014. **12**: p. 21.
209. Chu, L.-Y., D.P. Ramakrishnan, and R.L. Silverstein, *Thrombospondin-1 modulates VEGF signaling via CD36 by recruiting SHP-1 to VEGFR2 complex in microvascular endothelial cells.* Blood, 2013. **122**(10): p. 1822-1832.
210. Silverstein, R.L., et al., *Mechanisms of cell signaling by the scavenger receptor CD36: implications in atherosclerosis and thrombosis.* Trans Am Clin Climatol Assoc, 2010. **121**: p. 206-20.
211. Abumrad, N.A., et al., *Cloning of a rat adipocyte membrane protein implicated in binding or transport of long-chain fatty acids that is induced during preadipocyte differentiation. Homology with human CD36.* J Biol Chem, 1993. **268**(24): p. 17665-8.
212. Hirano, K., et al., *Pathophysiology of human genetic CD36 deficiency.* Trends Cardiovasc Med, 2003. **13**(4): p. 136-41.
213. Yamashita, S., et al., *Physiological and pathological roles of a multi-ligand receptor CD36 in atherogenesis; insights from CD36-deficient patients.* Mol Cell Biochem, 2007. **299**(1-2): p. 19-22.
214. Kar, N.S., et al., *Mapping and characterization of the binding site for specific oxidized phospholipids and oxidized low density lipoprotein of scavenger receptor CD36.* J Biol Chem, 2008. **283**(13): p. 8765-71.
215. Zhao, L., et al., *CD36 and lipid metabolism in the evolution of atherosclerosis.* British Medical Bulletin, 2018. **126**(1): p. 101-112.
216. Stewart, C.R., et al., *CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer.* Nat Immunol, 2010. **11**(2): p. 155-61.
217. Nath, A., et al., *Elevated free fatty acid uptake via CD36 promotes epithelial-mesenchymal transition in hepatocellular carcinoma.* Sci Rep, 2015. **5**: p. 14752.
218. Liang, Y., et al., *CD36 plays a critical role in proliferation, migration and tamoxifen-inhibited growth of ER-positive breast cancer cells.* Oncogenesis, 2018. **7**(12): p. 1-14.
219. Zhao, J., et al., *Exogenous lipids promote the growth of breast cancer cells via CD36.* Oncology Reports, 2017. **38**(4): p. 2105-2115.
220. Deng, M., et al., *CD36 promotes the epithelial–mesenchymal transition and metastasis in cervical cancer by interacting with TGF-β.* Journal of Translational Medicine, 2019. **17**(1): p. 352.
221. Luo, X., et al., *The fatty acid receptor CD36 promotes HCC progression through activating Src/PI3K/AKT axis-dependent aerobic glycolysis.* Cell Death & Disease, 2021. **12**(4): p. 328.

222. Nath, A., et al., *Elevated free fatty acid uptake via CD36 promotes epithelial-mesenchymal transition in hepatocellular carcinoma.* Scientific Reports, 2015. **5**(1): p. 14752.
223. Loh, C.Y., et al., *The E-Cadherin and N-Cadherin Switch in Epithelial-to-Mesenchymal Transition: Signaling, Therapeutic Implications, and Challenges.* Cells, 2019. **8**(10).
224. Peng, X., et al., *Apelin-13 induces MCF-7 cell proliferation and invasion via phosphorylation of ERK1/2.* International journal of molecular medicine, 2015. **36**(3): p. 733-738.
225. Caldon, C.E., et al., *Estrogen regulation of cyclin E2 requires cyclin D1 but not c-Myc.* Molecular and cellular biology, 2009. **29**(17): p. 4623-4639.
226. Wang, J. and Y. Li, *CD36 tango in cancer: signaling pathways and functions.* Theranostics, 2019. **9**(17): p. 4893-4908.
227. Molckovsky, A. and L.L. Siu, *First-in-class, first-in-human phase I results of targeted agents: highlights of the 2008 American society of clinical oncology meeting.* J Hematol Oncol, 2008. **1**: p. 20.
228. Jeanne, A., et al., *Original insights on thrombospondin-1-related antireceptor strategies in cancer.* Front Pharmacol, 2015. **6**: p. 252.
229. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.
230. Kim, J.T., et al., *Neurotensin, a novel target of Wnt/beta-catenin pathway, promotes growth of neuroendocrine tumor cells.* Int J Cancer, 2015. **136**(6): p. 1475-81.
231. Zaytseva, Y.Y., et al., *Cancer cell-associated fatty acid synthase activates endothelial cells and promotes angiogenesis in colorectal cancer.* Carcinogenesis, 2014. **35**(6): p. 1341-51.
232. Heuer, T.S., et al., *FASN Inhibition and Taxane Treatment Combine to Enhance Anti-tumor Efficacy in Diverse Xenograft Tumor Models through Disruption of Tubulin Palmitoylation and Microtubule Organization and FASN Inhibition-Mediated Effects on Oncogenic Signaling and Gene Expression.* EBioMedicine, 2017. **16**: p. 51-62.
233. Ventura, R., et al., *Inhibition of de novo Palmitate Synthesis by Fatty Acid Synthase Induces Apoptosis in Tumor Cells by Remodeling Cell Membranes, Inhibiting Signaling Pathways, and Reprogramming Gene Expression.* EBioMedicine, 2015. **2**(8): p. 806-22.
234. O'Farrell, M., et al., *Abstract 2675: Biomarker and PK/PD analyses of first in class FASN inhibitor TVB-2640 in a first-in-human phase 1 study in solid tumor patients.* AACR, 2015. **75**(15 (Suppl)).
235. Dean, E.J., et al., *Abstract 2512: Preliminary activity in the first in human study of the first-in-class fatty acid synthase (FASN) inhibitor, TVB-2640.* Journal of Clinical Oncology, 2016.
236. National Cancer Institute. *FASN Inhibitor TVB-2640 in Treating Patients with Colon or Other Cancers That Can Be Removed by Surgery*. 2017; Available from:

https://www.cancer.gov/about-cancer/treatment/clinical-trials/search/v?id=NCI-2016-01710&r=1.
237. Jafari, N., et al., *De Novo Fatty Acid Synthesis-Driven Sphingolipid Metabolism Promotes Metastatic Potential of Colorectal Cancer.* Mol Cancer Res, 2019. **17**(1): p. 140-152.
238. Glatz, J.F.C. and J. Luiken, *Dynamic role of the transmembrane glycoprotein CD36 (SR-B2) in cellular fatty acid uptake and utilization.* J Lipid Res, 2018. **59**(7): p. 1084-1093.
239. Glatz, J.F., J.J. Luiken, and A. Bonen, *Membrane fatty acid transporters as regulators of lipid metabolism: implications for metabolic disease.* Physiol Rev, 2010. **90**(1): p. 367-417.
240. Luiken, J.J., et al., *Post-translational modifications of CD36 (SR-B2): Implications for regulation of myocellular fatty acid uptake.* Biochim Biophys Acta, 2016. **1862**(12): p. 2253-2258.
241. Zhao, J., et al., *Exogenous lipids promote the growth of breast cancer cells via CD36.* Oncol Rep, 2017. **38**(4): p. 2105-2115.
242. Pan, J., et al., *CD36 mediates palmitate acid-induced metastasis of gastric cancer via AKT/GSK-3beta/beta-catenin pathway.* J Exp Clin Cancer Res, 2019. **38**(1): p. 52.
243. Watt, M.J., et al., *Suppressing fatty acid uptake has therapeutic effects in preclinical models of prostate cancer.* Sci Transl Med, 2019. **11**(478).
244. Menendez, J.A. and R. Lupu, *Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis.* Nat Rev Cancer, 2007. **7**(10): p. 763-77.
245. Altieri, D.C., *Targeting survivin in cancer.* Cancer Lett, 2013. **332**(2): p. 225-8.
246. Garg, H., et al., *Survivin: a unique target for tumor therapy.* Cancer Cell Int, 2016. **16**: p. 49.
247. DeBerardinis, R.J. and N.S. Chandel, *Fundamentals of cancer metabolism.* Sci Adv, 2016. **2**(5): p. e1600200.
248. Jafari, N., et al., *De Novo Fatty Acid Synthesis Driven Sphingolipid Metabolism Promotes Metastatic Potential of Colorectal Cancer.* Mol Cancer Res, 2018.
249. Silverstein, R.L. and M. Febbraio, *CD36, a scavenger receptor involved in immunity, metabolism, angiogenesis, and behavior.* Sci Signal, 2009. **2**(72): p. re3.
250. Julien, S., et al., *Characterization of a large panel of patient-derived tumor xenografts representing the clinical heterogeneity of human colorectal cancer.* Clin Cancer Res, 2012. **18**(19): p. 5314-28.
251. Cheung, A.F., et al., *Complete deletion of Apc results in severe polyposis in mice.* Oncogene, 2010. **29**(12): p. 1857-64.
252. Coort, S.L., et al., *Sulfo-N-succinimidyl esters of long chain fatty acids specifically inhibit fatty acid translocase (FAT/CD36)-mediated cellular fatty acid uptake.* Mol Cell Biochem, 2002. **239**(1-2): p. 213-9.
253. Sommer, K.W., et al., *Inhibitor of apoptosis protein (IAP) survivin is upregulated by oncogenic c-H-Ras.* Oncogene, 2003. **22**(27): p. 4266-80.

254. Rychahou, P., et al., *Colorectal cancer lung metastasis treatment with polymer-drug nanoparticles.* J Control Release, 2018. **275**: p. 85-91.
255. Sahin, A.A., et al., *Assessment of Ki-67-derived tumor proliferative activity in colorectal adenocarcinomas.* Mod Pathol, 1994. **7**(1): p. 17-22.
256. Ye, Q., et al., *ERK and AKT signaling cooperate to translationally regulate survivin expression for metastatic progression of colorectal cancer.* Oncogene, 2014. **33**(14): p. 1828-39.
257. Cao, D., et al., *Both de novo synthetized and exogenous fatty acids support the growth of hepatocellular carcinoma cells.* Liver Int, 2017. **37**(1): p. 80-89.
258. Cheng, C., et al., *Lipid metabolism reprogramming and its potential targets in cancer.* Cancer Commun (Lond), 2018. **38**(1): p. 27.
259. Clezardin, P., et al., *Expression of thrombospondin (TSP1) and its receptors (CD36 and CD51) in normal, hyperplastic, and neoplastic human breast.* Cancer Res, 1993. **53**(6): p. 1421-30.
260. Liang, Y., et al., *CD36 plays a critical role in proliferation, migration and tamoxifen-inhibited growth of ER-positive breast cancer cells.* Oncogenesis, 2018. **7**(12): p. 98.
261. Enciu, A.M., et al., *Targeting CD36 as Biomarker for Metastasis Prognostic: How Far from Translation into Clinical Practice?* Biomed Res Int, 2018. **2018**: p. 7801202.
262. Pascual, G., D. Dominguez, and S.A. Benitah, *The contributions of cancer cell metabolism to metastasis.* Dis Model Mech, 2018. **11**(8).
263. Showkat, M., M.A. Beigh, and K.I. Andrabi, *mTOR Signaling in Protein Translation Regulation: Implications in Cancer Genesis and Therapeutic Interventions.* Mol Biol Int, 2014. **2014**: p. 686984.
264. Altieri, D.C., *Survivin, cancer networks and pathway-directed drug discovery.* Nat Rev Cancer, 2008. **8**(1): p. 61-70.
265. Luengo, A., D.Y. Gui, and M.G. Vander Heiden, *Targeting Metabolism for Cancer Therapy.* Cell Chem Biol, 2017. **24**(9): p. 1161-1180.
266. Parca, L., et al., *Modeling cancer drug response through drug-specific informative genes.* Sci Rep, 2019. **9**(1): p. 15222.
267. Ahmed, D., et al., *Epigenetic and genetic features of 24 colon cancer cell lines.* Oncogenesis, 2013. **2**: p. e71.
268. Park, J., et al., *Obesity and cancer--mechanisms underlying tumour progression and recurrence.* Nat Rev Endocrinol, 2014. **10**(8): p. 455-465.
269. Hellmann, J., et al., *Increased saturated fatty acids in obesity alter resolution of inflammation in part by stimulating prostaglandin production.* J Immunol, 2013. **191**(3): p. 1383-92.
270. Berndt, J., et al., *Fatty acid synthase gene expression in human adipose tissue: association with obesity and type 2 diabetes.* Diabetologia, 2007. **50**(7): p. 1472-80.
271. Balaban, S., et al., *Obesity and cancer progression: is there a role of fatty acid metabolism?* Biomed Res Int, 2015. **2015**: p. 274585.

272. Drover, V.A., et al., *CD36 mediates both cellular uptake of very long chain fatty acids and their intestinal absorption in mice.* J Biol Chem, 2008. **283**(19): p. 13108-15.
273. Jayawardhana, A., et al., *Fatty acid-like Pt(IV) prodrugs overcome cisplatin resistance in ovarian cancer by harnessing CD36.* Chem Commun (Camb), 2020. **56**(73): p. 10706-10709.
274. Drury, J., et al., *Inhibition of Fatty Acid Synthase Upregulates Expression of CD36 to Sustain Proliferation of Colorectal Cancer Cells.* Front Oncol, 2020. **10**: p. 1185.
275. Deng, M., et al., *CD36 promotes the epithelial-mesenchymal transition and metastasis in cervical cancer by interacting with TGF-beta.* J Transl Med, 2019. **17**(1): p. 352.
276. Yang, P., et al., *Dietary oleic acid-induced CD36 promotes cervical cancer cell growth and metastasis via up-regulation Src/ERK pathway.* Cancer Lett, 2018. **438**: p. 76-85.
277. Kalluri, R. and E.G. Neilson, *Epithelial-mesenchymal transition and its implications for fibrosis.* J Clin Invest, 2003. **112**(12): p. 1776-84.
278. Kalluri, R. and R.A. Weinberg, *The basics of epithelial-mesenchymal transition.* J Clin Invest, 2009. **119**(6): p. 1420-8.
279. Zhang, Y.W., R., *Epithelial-to-mesenchymal transition in cancer: complexity and opportunities.* Frontiers in Medicine, 2018. **12**(361–373(2018)).
280. Polyak, K. and R.A. Weinberg, *Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits.* Nat Rev Cancer, 2009. **9**(4): p. 265-73.
281. Ocana, O.H., et al., *Metastatic colonization requires the repression of the epithelial-mesenchymal transition inducer Prrx1.* Cancer Cell, 2012. **22**(6): p. 709-24.
282. Pecina-Slaus, N., *Tumor suppressor gene E-cadherin and its role in normal and malignant cells.* Cancer Cell Int, 2003. **3**(1): p. 17.
283. Yun, J.A., et al., *Loss of E-Cadherin expression is associated with a poor prognosis in stage III colorectal cancer.* Oncology, 2014. **86**(5-6): p. 318-28.
284. Christou, N., et al., *E-cadherin: A potential biomarker of colorectal cancer prognosis.* Oncol Lett, 2017. **13**(6): p. 4571-4576.
285. Okugawa, Y., et al., *Clinical significance of serum soluble E-cadherin in colorectal carcinoma.* J Surg Res, 2012. **175**(2): p. e67-73.
286. Wang, R., et al., *The Characteristics and Prognostic Effect of E-Cadherin Expression in Colorectal Signet Ring Cell Carcinoma.* PLoS One, 2016. **11**(8): p. e0160527.
287. Gao, M., et al., *Expression analysis and clinical significance of eIF4E, VEGF-C, E-cadherin and MMP-2 in colorectal adenocarcinoma.* Oncotarget, 2016. **7**(51): p. 85502-85514.
288. Kitadai, Y., et al., *In situ mRNA hybridization technique for analysis of metastasis-related genes in human colon carcinoma cells.* Am J Pathol, 1995. **147**(5): p. 1238-47.

289. Reinhold, W.C., et al., *Multifactorial regulation of E-cadherin expression: an integrative study.* Mol Cancer Ther, 2010. **9**(1): p. 1-16.
290. Jablonska-Trypuc, A., M. Matejczyk, and S. Rosochacki, *Matrix metalloproteinases (MMPs), the main extracellular matrix (ECM) enzymes in collagen degradation, as a target for anticancer drugs.* J Enzyme Inhib Med Chem, 2016. **31**(sup1): p. 177-183.
291. Hrabec, E., et al., *[Type IV collagenases (MMP-2 and MMP-9) and their substrates--intracellular proteins, hormones, cytokines, chemokines and their receptors].* Postepy Biochem, 2007. **53**(1): p. 37-45.
292. Illman, S.A., et al., *Epilysin (MMP-28) induces TGF-β mediated epithelial to mesenchymal transition in lung carcinoma cells.* Journal of Cell Science, 2006. **119**(18): p. 3856-3865.
293. Jian, P., et al., *MMP28 (epilysin) as a novel promoter of invasion and metastasis in gastric cancer.* BMC Cancer, 2011. **11**: p. 200.
294. Zhang, J., et al., *Overexpression of MMP21 and MMP28 is associated with gastric cancer progression and poor prognosis.* Oncol Lett, 2018. **15**(5): p. 7776-7782.
295. Illman, S.A., et al., *Epilysin (MMP-28) induces TGF-beta mediated epithelial to mesenchymal transition in lung carcinoma cells.* J Cell Sci, 2006. **119**(Pt 18): p. 3856-65.
296. Illman, S.A., J. Lohi, and J. Keski-Oja, *Epilysin (MMP-28)--structure, expression and potential functions.* Exp Dermatol, 2008. **17**(11): p. 897-907.
297. Ferber, E.C., et al., *A role for the cleaved cytoplasmic domain of E-cadherin in the nucleus.* J Biol Chem, 2008. **283**(19): p. 12691-700.
298. McCusker, C.D. and D. Alfandari, *Life after proteolysis: Exploring the signaling capabilities of classical cadherin cleavage fragments.* Commun Integr Biol, 2009. **2**(2): p. 155-7.
299. Tseng, W., X. Leong, and E. Engleman, *Orthotopic mouse model of colorectal cancer.* J Vis Exp, 2007(10): p. 484.
300. Agarwal, E., M.G. Brattain, and S. Chowdhury, *Cell survival and metastasis regulation by Akt signaling in colorectal cancer.* Cell Signal, 2013. **25**(8): p. 1711-9.
301. Pan, J., et al., *CD36 mediates palmitate acid-induced metastasis of gastric cancer via AKT/GSK-3β/β-catenin pathway.* Journal of Experimental & Clinical Cancer Research, 2019. **38**(1): p. 52.
302. Zhang, Y. and R.A. Weinberg, *Epithelial-to-mesenchymal transition in cancer: complexity and opportunities.* Front Med, 2018. **12**(4): p. 361-373.
303. Radisky, E.S. and D.C. Radisky, *Matrix metalloproteinase-induced epithelial-mesenchymal transition in breast cancer.* J Mammary Gland Biol Neoplasia, 2010. **15**(2): p. 201-12.
304. Egeblad, M. and Z. Werb, *New functions for the matrix metalloproteinases in cancer progression.* Nature Reviews Cancer, 2002. **2**(3): p. 161-174.
305. Lochter, A., et al., *Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular alterations that leads to stable epithelial-to-mesenchymal conversion*

and a premalignant phenotype in mammary epithelial cells. J Cell Biol, 1997. **139**(7): p. 1861-72.
306. Maretzky, T., et al., *ADAM10 mediates E-cadherin shedding and regulates epithelial cell-cell adhesion, migration, and beta-catenin translocation.* Proc Natl Acad Sci U S A, 2005. **102**(26): p. 9182-7.
307. Torre, L.A., et al., *Global cancer statistics, 2012.* CA Cancer J Clin, 2015. **65**(2): p. 87-108.
308. Hisamuddin, I.M. and V.W. Yang, *Genetics of colorectal cancer.* MedGenMed, 2004. **6**(3): p. 13.
309. Nathke, I.S., *The adenomatous polyposis coli protein: the Achilles heel of the gut epithelium.* Annu Rev Cell Dev Biol, 2004. **20**: p. 337-66.
310. McCart, A.E., N.K. Vickaryous, and A. Silver, *Apc mice: Models, modifiers and mutants.* Pathology - Research and Practice, 2008. **204**(7): p. 479-490.
311. Halberg, R.B., et al., *Tumorigenesis in the multiple intestinal neoplasia mouse: redundancy of negative regulators and specificity of modifiers.* Proc Natl Acad Sci U S A, 2000. **97**(7): p. 3461-6.
312. McCart, A.E., N.K. Vickaryous, and A. Silver, *Apc mice: models, modifiers and mutants.* Pathol Res Pract, 2008. **204**(7): p. 479-90.
313. Byun, A.J., et al., *Colon-specific tumorigenesis in mice driven by Cre-mediated inactivation of Apc and activation of mutant Kras.* Cancer Lett, 2014. **347**(2): p. 191-5.
314. Hagland, H.R. and K. Soreide, *Cellular metabolism in colorectal carcinogenesis: Influence of lifestyle, gut microbiome and metabolic pathways.* Cancer Lett, 2015. **356**(2 Pt A): p. 273-80.
315. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
316. Hart, G.W., *Glycosylation.* Curr Opin Cell Biol, 1992. **4**(6): p. 1017-23.
317. Carrillo, L.D., J.A. Froemming, and L.K. Mahal, *Targeted in vivo O-GlcNAc sensors reveal discrete compartment-specific dynamics during signal transduction.* J Biol Chem, 2011. **286**(8): p. 6650-8.
318. McKnight, G.L., et al., *Molecular cloning, cDNA sequence, and bacterial expression of human glutamine:fructose-6-phosphate amidotransferase.* J Biol Chem, 1992. **267**(35): p. 25208-12.
319. Hart, G.W., M.P. Housley, and C. Slawson, *Cycling of O-linked beta-N-acetylglucosamine on nucleocytoplasmic proteins.* Nature, 2007. **446**(7139): p. 1017-22.
320. Kreppel, L.K., M.A. Blomberg, and G.W. Hart, *Dynamic glycosylation of nuclear and cytosolic proteins. Cloning and characterization of a unique O-GlcNAc transferase with multiple tetratricopeptide repeats.* J Biol Chem, 1997. **272**(14): p. 9308-15.
321. Dennis, J.W., M. Granovsky, and C.E. Warren, *Glycoprotein glycosylation and cancer progression.* Biochim Biophys Acta, 1999. **1473**(1): p. 21-34.
322. Slawson, C. and G.W. Hart, *O-GlcNAc signalling: implications for cancer cell biology.* Nat Rev Cancer, 2011. **11**(9): p. 678-84.

323. Venkitachalam, S., et al., *Biochemical and functional characterization of glycosylation-associated mutational landscapes in colon cancer.* Sci Rep, 2016. **6**: p. 23642.
324. Oliveira-Ferrer, L., K. Legler, and K. Milde-Langosch, *Role of protein glycosylation in cancer metastasis.* Semin Cancer Biol, 2017. **44**: p. 141-152.
325. Rodrigues, J.G., et al., *Glycosylation in cancer: Selected roles in tumour progression, immune modulation and metastasis.* Cell Immunol, 2018. **333**: p. 46-57.
326. William James, A., et al., *Crosstalk between protein N-glycosylation and lipid metabolism in Saccharomyces cerevisiae.* Scientific Reports, 2019. **9**(1).
327. Lyons, S.D., M.E. Sant, and R.I. Christopherson, *Cytotoxic mechanisms of glutamine antagonists in mouse L1210 leukemia.* Journal of Biological Chemistry, 1990. **265**(19): p. 11377-11381.
328. Tamm, I., et al., *IAP-Family Protein Survivin Inhibits Caspase Activity and Apoptosis Induced by Fas (CD95), Bax, Caspases, and Anticancer Drugs.* Cancer Research, 1998. **58**(23): p. 5315-5320.
329. Malinowsky, K., et al., *Activation of the PI3K/AKT pathway correlates with prognosis in stage II colon cancer.* Br J Cancer, 2014. **110**(8): p. 2081-9.
330. Fang, Y., et al., *CD36 inhibits β-catenin/c-myc-mediated glycolysis through ubiquitination of GPC4 to repress colorectal tumorigenesis.* Nature Communications, 2019. **10**(1): p. 3981.
331. Feng, W.W., et al., *CD36-Mediated Metabolic Rewiring of Breast Cancer Cells Promotes Resistance to HER2-Targeted Therapies.* Cell Rep, 2019. **29**(11): p. 3405-3420 e5.
332. Feng, W.W., S. Bang, and M. Kurokawa, *CD36: a key mediator of resistance to HER2 inhibitors in breast cancer.* Molecular & Cellular Oncology, 2020. **7**(2): p. 1715766.
333. Jin, Q., et al., *Fatty acid synthase phosphorylation: a novel therapeutic target in HER2-overexpressing breast cancer cells.* Breast Cancer Res, 2010. **12**(6): p. R96.
334. Dolgin, E., *A drug to block fat intake and combat cancer spread.* Nature Outlook: The Spinoff Prize 2021, 2021.
335. Giles, R.H., J.H. van Es, and H. Clevers, *Caught up in a Wnt storm: Wnt signaling in cancer.* Biochim Biophys Acta, 2003. **1653**(1): p. 1-24.
336. Madison, B.B., *Srebp2: A master regulator of sterol and fatty acid synthesis.* J Lipid Res, 2016. **57**(3): p. 333-5.
337. Antoniewicz, M.R., *A guide to 13C metabolic flux analysis for the cancer biologist.* Experimental & Molecular Medicine, 2018. **50**(4): p. 1-13.
338. Pan, J., et al., *CD36 mediates palmitate acid-induced metastasis of gastric cancer via AKT/GSK-3β/β-catenin pathway.* Journal of Experimental & Clinical Cancer Research, 2019. **38**(1).
339. Fanjul-Fernández, M., et al., *Matrix metalloproteinases: Evolution, gene regulation and functional analysis in mouse models.* Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2010. **1803**(1): p. 3-19.

340. Overall, C.M. and C. López-Otín, *Strategies for MMP inhibition in cancer: innovations for the post-trial era.* Nature Reviews Cancer, 2002. **2**(9): p. 657-672.
341. Kim, E.-S., M.-S. Kim, and A. Moon, *TGF-β-induced upregulation of MMP-2 and MMP-9 depends on p38 MAPK, but not ERK signaling in MCF10A human breast epithelial cells.* International journal of oncology, 2004. **25**(5): p. 1375-1382.
342. Andres, D.A., et al., *Improved workflow for mass spectrometry-based metabolomics analysis of the heart.* J Biol Chem, 2020. **295**(9): p. 2676-2686.
343. Brewer, M.K., et al., *Targeting pathogenic Lafora bodies in Lafora disease using an antibody-enzyme fusion.* Cell metabolism, 2019. **30**(4): p. 689-705. e6.
344. Sun, R.C., et al., *Nuclear glycogenolysis modulates histone acetylation in human non-small cell lung cancers.* Cell metabolism, 2019. **30**(5): p. 903-916. e7.
345. Fiehn, O., *Metabolomics by gas chromatography–mass spectrometry: combined targeted and untargeted profiling.* Current protocols in molecular biology, 2016. **114**(1): p. 30.4. 1-30.4. 32.
346. Kind, T., et al., *FiehnLib: mass spectral and retention index libraries for metabolomics based on quadrupole and time-of-flight gas chromatography/mass spectrometry.* Analytical chemistry, 2009. **81**(24): p. 10038-10048.
347. Zhang, L., et al., *Serial dilution curve: a new method for analysis of reverse phase protein array data.* Bioinformatics, 2009. **25**(5): p. 650-4.
348. *The Cancer Proteome Atlas*. [cited 2021; Available from: https://www.tcpaportal.org/tcpa/faq.html.
349. Neeley, E.S., et al., *Variable slope normalization of reverse phase protein arrays.* Bioinformatics, 2009. **25**(11): p. 1384-9.

VITA

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EDUCATION

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ACADEMIC HONORS AND AWARDS

Yulan Sun Outstanding Graduate Student Award	2021
Molecular Mechanisms in Toxicology T32 Trainee	2018 – 2020
Judges Choice Award Winner – Von Allmen Center for Entrepreneurship (VACE) "60 Second Elevator Pitch Competition"	2019
Office of Biomedical Education Travel Award	2019
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POSITIONS HELD

Graduate Student Congress Representative – Department of Toxicology and Cancer Biology	2019 – 2021

NATIONAL PRESENTATIONS

James M. Drury, James M. Drury, Piotr Rychahou, Heidi Weiss and Yekaterina Y. Zaytseva. CD36, a fatty acid translocase, promotes metastasis in CRC. AACR Virtual Meeting, May 17-21, 2021

James M. Drury, James M. Drury, Piotr Rychahou, Heidi Weiss and Yekaterina Y. Zaytseva. Upregulation of CD36 is a mechanism of resistance to FASN-targeted therapy in colorectal cancer. AACR Virtual Meeting II, June 22-24, 2020

James M. Drury, Naser Jafari, Piotr G. Rychahou, Tianyan Gao, Mark Evers, Yekaterina Y. Zaytseva. CD36 in colorectal cancer. AACR Annual Meeting, March 29 - April 3, 2019, Atlanta, Georgia.

James M. Drury, Naser Jafari, Yekaterina Y. Zaytseva. Overexpression of CD36/fatty acid translocase promotes tumorigenesis in colorectal cancer. AACR Annual Meeting, April 14-18, 2018, Chicago, Illinois

PUBLICATIONS

James Drury, Piotr G. Rychahou, Daheng He, Naser Jafari1, Chi Wang, Eun Y. Lee, Heidi L. Weiss, Bernard Mark Evers and Yekaterina Y. Zaytseva. "Inhibition of Fatty Acid Synthase Upregulates Expression of CD36 to Sustain Proliferation of Colorectal Cancer Cells." Frontiers in Oncology. 2020. PMID: 32850342

Naser Jafari, **James M. Drury**, Andrew Morris, Fredrick O. Onono, Payton D. Stevens, Tianyan Gao, Eun Lee, Heidi Weiss, B. Mark Evers and Yekaterina Y. Zaytseva. De Novo Fatty Acid Synthesis Driven Sphingolipid Metabolism Promotes Metastatic Potential of Colorectal Cancer. Molecular Cancer Research. 2018. PMID: 30154249.

MANUSCRIPTS IN PREPARATION

James Drury, Piotr G. Rychahou, Courtney Kelson, Mariah Geisen, Yuanyuan Wu, Daheng He, Chi Wang, Eun Y Lee, Mark B Evers, and Yekaterina Y. Zaytseva. CD36, a fatty acid translocase, promotes metastasis via upregulation MMP28 and an increase in e-cadherin cleavage in colorectal cancer.

James Drury, Young LE, He D, Wu Y, Wang C, Weiss HL, Sun R, and Zaytseva YY. Tissue-specific deletion of Fatty Acid Synthase is associated with increased survival and suppressed intestinal tumor development in the mouse model of Apc-driven colorectal cancer.

James Drury, Young LE, Geisen M, Kelson CO, He D, Wu Y, Wang C, Sun R, and Zaytseva YY. Overexpression of Fatty Acid Synthase upregulates Glutamine--Fructose-6-Phosphate Transaminase 1 and increases O-GlcNAc protein glycosylation to promote colorectal cancer growth.