REGULATION OF COLON CANCER GROWTH VIA LOSS OF FOXO3-MEDIATED INCREASEDACYLTRANSFERASE (DGATs) ENZYMES

AN ABSTRACT

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BY

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ABSTRACT

Colon cancer is currently the 2nd most common cause of cancer related deaths in the USA but it is the number one cause of cancer for individuals who are non-smokers in the United States. Recently, there have been changing trends that display a younger age demographic being diagnosed with sporadic colon cancer. Moreover, southern states such as Louisiana currently demonstrate the highest rates of colorectal cancer cases in comparison to other areas in the USA. While the reasons for these changing trends has not been established, recent findings have depicted a link between loss of FOXO3 function and increased lipid droplet (LD) accumulation in non-adipose tissue such as the colon which can act as energy for tumor metabolic and proliferative needs. FOXO3, which is a transcription factor and tumor suppressor, is inactivated in colon cancer. However, the link between loss of FOXO3 function and increased LDs remains to be fully understood. One possible connection could be Acyltransferase DGATs enzymes that are involved in the final stage of triglyceride synthesis (lipogenesis), yet the specific mechanisms of this regulation are unknown. We found that DGAT1/2 inhibition protected against loss of FOXO3 after oleic acid-mediated increases in LD density, indicating a critical role for DGATs in mediating the FOXO3-LD lipogenic network. Furthermore, we found that inhibition of DGAT1/2 prevented upstream PI3K-Akt induced phosphorylation and subsequent loss of FOXO3, suggesting a regulatory loop between DGATs and the PI3K-FOXO3 axis. Functionally, we observed that inhibition of DGATs rescued expression of the FOXO3 downstream transcriptional target, p27kip1, which is typically lost after treatment of oleic acid. Together these data indicate a critical role for DGATs in mediating
the lipogenic effects of FOXO3-LDs and suggests DGATs as a therapeutic target that could restore FOXO3 tumor suppressor function. Further understanding of the mechanism that governs DGATs in relation to FOXO3 and LDs in colon cancer is necessary, to establish their potential role as a therapeutic target and/or biomarker.

*Keywords*: Colon Cancer, FOXO3, DGATs, Lipid Droplet
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INTRODUCTION

**Epidemiology:** Cancer is currently the second leading cause of death in the United States, with the four major cancers being lung, breast, prostate, and colorectal. Colon cancer is the third most commonly occurring cancer worldwide and the fourth most frequent cause of death that has an oncological origin. Furthermore, it is the third most common cancer in men and the second most common cancer in women. There were 1,096,601 new cases of colon cancer and over half a million deaths in 2018 alone. Global statistics combine cancers of the colon, rectum, and anus under the category of colorectal cancer, which is the most common gastrointestinal neoplasia. (1)

Although colon cancer is the second most common cause of cancer in the United States, it is the number one cause of cancer for people who are non-smokers in the United States. The overall instance of colon and rectal cancer cases each year is between 125,000-150,000 people. As of 2016, there were approximately 1 million people living with colon and rectal cancer in the United States. Additionally, these 1 million people included colon cancer cases that were in all four different stages of the disease. (2)

Invasive cancer accounts for 39.3% of diagnoses in men and 37.7% of diagnoses in women. Although these gender differences are not fully understood, the fact remains that colon cancer is among the top three most common cancers seen in both genders. Furthermore, cancer statistics have estimated that there will be 101,420 new cases of colon cancer and 51,020 associated deaths in 2019 for both genders in the United States. As a
Aside note to these national estimates, 2,340 of new colorectal cancer cases are expected to occur in Louisiana specifically. (3-4)

The global incidence rates for colon cancer in 2018 were highest in Europe, Australia/New Zealand, North America, and Eastern Asia. Furthermore, the rates of colon cancer incidence have increased equally among both genders in developing countries such as Uruguay and sub-Saharan Africa. (1) Since the early 2,000’s there has been an increase in the incidence and/or mortality for CRC among people that are younger than 50 years of age. This upward trend in a younger age of onset has become of particular concern for countries such as the United States, Canada, Australia, and Norway as of 2017. (5-6)

Fluctuation patterns in age demographic and geographical trends, both in the USA and globally, for colon cancer have been reported. In 2000, only 21% of this age demographic were being screened whereas 60% of this age demographic were screened in 2015. (7) In parallel to the changes in screening patterns that have been noted, there have also been drastic changes in the American geographical patterns of CRC over time. States that are situated in the deep South and Midwest currently have the highest rates of CRC. However, the Northeastern states used to have the highest rates of CRC during the 1970’s and 1980’s. (8)

In the past two decades, countries that are part of the European Union have instituted organized screenings for people above the age of 50. (9) Despite increased screenings, CRC accounted for the second highest number of deaths among EU countries in 2018 and totaling 177,400 mortalities. In particular, Poland and Spain had the highest mortality rates of CRC out of all the countries in EU. In comparison to the above mentioned upward trend for CRC in people that are below the age of 50, the EU does not appear to be
demonstrating this same trend. Based on an analysis of both the World Health Organization (WHO) and Eurostat databases, the EU does not predict an upward spike in colorectal cancer among people younger than 50 in 2019. (10)

**Etiology:** In both sporadic and familial cases, several different genes have been identified as being associated with the underlying etiology of colorectal cancer. Moreover, transcription factor dysregulation and the resultant changes in gene expression are significant factors of tumor cell differentiation, proliferation, migration, metastatic abilities, and resistance to chemotherapeutic treatment agents. (11)

**Risk Factors:** When looking at colon cancer risk factors, we know of certain things that increase risk, perhaps most importantly family history and first-degree relatives. In some cases, colon cancer is clearly associated with inherited cancer syndromes. In other cases, families have an increased risk for unknown reasons. Inflammatory diseases of the colon, such as ulcerative colitis and Crohn’s disease, clearly have an increased risk of colon cancer development. Physical activity can, in part, be protective in preventing colon cancer. (12) Additional colon cancer risk factors that are known to increase occurrence include diabetes, obesity, western diet of red meat and low fiber, as well as smoking and alcohol consumption. A diet high in red or processed meats has been associated with an increased risk of colon cancer, but to lesser of an extent has been associated with rectal cancer. (13) Mice that have been on a high-fat diet also had an increased predisposition to developing colon cancer. These findings illustrate an important link that exists between obesity, lipid droplet accumulation, and colorectal cancer development. However, the complete mechanism of these processes are not fully understood. (14)
During a colon cancer risk assessment, there are certain aspects of the family history that should be focused on. If there is a personal family history of colon, endometrial, or breast cancer prior to the age of 50 years old, this is considered an early age diagnosis and raises red flags. Additionally, if an individual has 10 or more cumulative adenomatous colon polyps or if a family history of someone having had these characteristics exists. Similarly, ovarian cancer or male breast cancer, as well as multiple primary cancers in an individual are warning signs of an increased risk to develop colon cancer. Lastly, if there is a history of multiple relatives and multiple generations that were affected. (15)

**Categories/Classification:** Colon cancer can be classified into three main categories which consist of sporadic cases, hereditary, and familial. A majority of colon cancers are sporadic, which means that they have nothing to do with inherited genetics but rather they are happening completely by chance. Only about 5-10% of colon cancer cases have a hereditary cause, which means that it happens because of a single underlying genetic mutation that increases the risk. Although there is an increased risk of colon cancer, there may also be an increased risk for other cancers, depending on which gene is involved. Lynch syndrome is the most common hereditary cause of colon cancer. In familial cases, a hereditary cause was ruled out, but there is more than a fair share of colon cancer that runs in the family. However, there is not a way to genetically test these individuals because the specific underlying cause of increased colon cancer in their family history is not currently known. (16, 17, 18)

Familial Adenomatous Polyposis (FAP), also known as Classic FAP, is caused by a mutation on the APC gene. Clinically FAP presents with an increased number of adenomatous polyps throughout the colon at an early age, characteristically in an individual
that is 18-20 years of age. As a result of this early age of polyp occurrence, annual colonoscopy screenings should begin during the early tween years. (19-20)

Attenuated Familial Adenomatous Polyposis (AFAP) is caused by a mutation that is located at codon 1309 on the APC gene. Although there are usually less than 100 polyps seen in these patients, the primary difference between FAP and AFAP is the location of the polyps. Attenuated-FAP will have an increased number of polyps in the ascending, transverse, and descending colon. However, there are rarely any polyps found in the rectum. For this reason, these individuals are eligible to have an ileorectal anastomosis (IRA) rather than a total colectomy. These individuals are also at an increased risk of desmoid disease, which is the rapid build-up of mesenchymal soft tissue in atypical regions of the body. If intra-abdominal desmoids are not removed as soon as possible, eventually they will grow around the colon and cause obstructions. Surgical attempts to remove desmoids will commonly result in death among these patients due to the development of strictures that cause colonic ischemia. (21-22)

Hereditary Nonpolyposis Colorectal Cancer (HNPCC) is now referred to as Lynch Syndrome and is caused by a mutation in one of the mismatch repair (MMR) genes, specifically MSH2, MSH6, MLH1, PMS1, and PMS2. Each of these genetic mutations correlates to an increased risk of colon cancer development. For MLH1 and MSH2 there is a 40-80% chance, for MSH6 there is a 10-22% chance, and for PMS1 and PMS2 there is a 15-20% chance of colon cancer development. Individuals with lynch syndrome will classically present with an increased amount of colon polyps between the age of 20-40 years old. Evidence has recently shown that there is accelerated colonic adenoma to colorectal carcinoma occurrence in those that have lynch syndrome. (23-24)
Pathogenesis and Carcinogenesis of Colon Cancer: The Adenoma-Carcinoma sequence is related to a sequence of specific genes; APC, DCC is deleted in colon cancer, p53, etc. Hyperplastic Polyp → Adenoma → Sessile Adenoma → Cancer. This mechanism is the way in which we thought all tumors developed for a long time but now we are finding out that only about 60% of all colon cancers follow this initial pathway and about 40% of colon cancers follow a completely different pathway of carcinogenesis. Currently, there are 2 or 3 majorly proposed pathways that we are aware of, including the Serrated-Adenoma Pathway and the Lynch Promoter Pathway. Serrated adenomas are a type of polyp that have been recognized for following a completely different pathway of carcinogenesis that involves hypermethylation of promoter genes. The traditional adenoma-carcinoma sequence was believed to take 5-10 years for colon cancer development to occur. However, the more recently understood pathways can develop a tumor in 1-2 years and this might have something to do with why we are seeing a rise in sporadic colorectal cancer among a younger age group. Currently the southern USA is leading in the number of cases with under age colon and rectal cancer. Moreover, southern states rank in the top 25% of national overall per capita cases of colon and rectal cancer. The carcinogenesis of colorectal cancer has been associated with microsatellite instability (MSI), chromosomal instability (CIN), and a CpG island methylator phenotype (CIMP). These three molecular pathways demonstrate patterns of genetic instability. (25-27)

Staging/Prognostic Factors: As a rule of thumb, cancer has three main aspects: name, stage, treatment. For solid tumors staging is almost always done the same way; invasion of local tissue, lymph node involvement, and metastases. Treatment usually consists of three possible options; surgery, radiation, chemotherapy. The staging (Table 1)
of colon cancer determines which of these treatment options will be implemented. If there is only local invasion of the mucosa or submucosal layers, it is classified as Stage-I colon cancer. If there is invasion through the deeper serosa or muscularis layers, it is classified as Stage-II colon cancer. If invasion has occurred through the entire wall of the colon, it is classified as being Stage-IIb colon cancer. Once the lymph nodes are involved, it is classified as being Stage-III colon cancer and if there is metastasis to other organs, it is classified as being Stage-IV colon cancer. Since there are different treatment options, determining the stage prior to colon surgery is a necessity for proper management. Initially a CAT scan and torso scan with liver protocol is done because the most common location of colorectal cancer metastasis is the liver. The rectum is divided into 3 segments, each of which is correlated with nodal metastases that varies depending on the segment involved. Colorectal cancer that is in the upper segment of the rectum will commonly metastasize to the liver, while cancer that is in the middle rectum will commonly metastasize to the inguinal lymph nodes as well as the liver. Inguinal lymph node metastasis that does not involve the liver is associated with cancer in the lower segment of the rectum. One of the ways in which these individuals are staged, in addition to a pelvic scan, would be to have imaging done via an MRI of the pelvis. Initially this was done with an anal ultrasound but this technique has been replaced with pelvic scans, which determines whether there is sphincter involvement. Another way in which staging can be accomplished is by use of biomarkers, such as CMP or CEA. The tumor marker for colon cancer is CEA, which is much more useful for recurrence of colon cancer but can also be used during initial diagnostics. In a patient with colon cancer, you might see a CEA level that is between 4-5 but this level will drop after surgical resection. However, if the CEA level begins to
increase on follow-up appointments, this would imply that there is an occult metastatic disease present. (25, 28, 29-32)

<table>
<thead>
<tr>
<th>STAGING</th>
<th>FEATURES</th>
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| Stage I | No tumor growth through the muscle layer  
No lymph node involvement | |
| Stage II | Tumor has grown through the muscle layer  
No lymph node involvement | |
| Stage III | Tumor is found in the resected lymph nodes  
Lymph nodes are positive | Regardless of how far into the wall tumor has grown, if the lymph nodes are positive, it is classified as stage III disease |
| Stage IV | Colon cancer has spread from the place of origin and is now found in other organs of the body  
Most common site of metastasis is the liver | |

Table 1. Staging and features of Colon Cancer

Treatment Options: Treatment for Stage I or IIa will be surgery. Patients with Stage IIb or III will also have surgical treatment as the first course of action, however they will additionally have chemotherapy post-surgery. Stage IV disease will have chemotherapy prior to surgery, in order to shrink the tumor. Since 1992 the American College of Gastroenterology (ACG) has stated that 12 lymph nodes must be involved to have adequate pathology of colorectal cancer. The reasoning behind this number is because it is believed that 12 lymph nodes will provide adequate sampling to determine if a patient has nodal metastatic disease. In addition to staging, treatment will also depend on where the cancer is located in the colon because watershed areas and redundant blood flow will impact the possibilities of resection. (29)

FOLFOX is a combination of chemotherapy drugs that consists of 5-Flourouracil (5FU), Leucovorin, and Oxaliplatin. 5FU can be administered in two different ways, either
intravenously (IV) or it can be given orally. Xeloda is the tradename of orally administered 5FU, which is given to patients that are receiving pre-surgical radiation therapy because xeloda is broken down into the same metabolite as 5FU. Leucovorin and Oxaliplatin can only be given intravenously. The second line of chemotherapy treatment for colorectal cancer is FOLFIRI. In patients with stage IV tumors that respond to anti-VEGF, Avastin can be used but they cannot be operated on because the patient will not heal. Monoclonal antibody therapies that target KRAS, can be used to in patients with metastatic disease because 45-50% of these patients are KRAS wild-type. However, patients that have metastatic disease and are KRAS-mutant, are not eligible for this monoclonal antibody option because they will not respond to the treatment. (30-31)

**Targeted Therapies:** Two main categories of targeted therapies exist for colon cancer/colorectal cancer. VEGF targeted therapies have to do with blood supply to the tumors. There are four main types that include Bevacizumab, Afibercept, Ramucinumab, and Regorafenib which is a multi-kinase inhibitor. The first three agents are given along with chemotherapy and the last one listed is administered on its own. EGFR targeted therapies are the second category. Tumor mutations in KRAS/NRAS genes render this treatment ineffective so these drugs will only work if the tumor doesn’t have these genetic mutations. They include Cetuximab and Panitumumab, which can be given as a single agent or along with chemotherapy treatment. (29-31, 32)
PART I. OVERVIEW

**FOXOs:** The Forkhead Box O (FOXO) family of transcription factors encompass four known subtypes in humans, referred to as FOXO1 (FKHR), FOXO3 (FKHRL1), FOXO4 (AFX1), and FOXO6. Although FOXO6 expression is restricted to central nervous system tissue, the other FOXO family members are expressed in a universal manner among all human tissues. All members of the FOXO family are characterized as having an evolutionarily conserved ‘Forkhead-box’ DNA-binding domain that is wing-shaped. This DNA-binding domain specifically binds to 5’-TTGTTTAC-3’ DNA sequences. It is through this DNA-binding process that the FOXO family of transcription factors are able to elicit their transcriptional activity in human cells. (33)

Additionally, there is a shuttling mechanism that plays a large role in regulating the transcriptional activity of FOXOs. While their nuclear location permits binding to DNA in promoter and enhancer regions, which correlates with active transcription occurring, they are unable to carry out this function if they become re-located to the cytoplasm. If FOXOs are phosphorylated on three of their RxRxxS/T conserved residues, the 14-3-3 regulator proteins are able to bind directly to the FOXOs and facilitate the process of nuclear export via this shuttling process. This process is predominantly correlated with the FOXO1, FOXO3, and FOXO4 subtypes. Upon re-location to the cytoplasm, these FOXOs remain inactivated by the sustained phosphorylation of their RxRxxS/T residues as well as being unable to re-enter the nucleus due to the persistent binding of regulator proteins 14-3-3. (34)
The FOXO family of transcription factors are classified as being tumor suppressors because they have an inhibitory effect on cancer cell growth and survival. In the nucleus, the FOXO family transcribe a variety of target genes that are involved in processes such as cell cycle regulation, apoptosis, and angiogenesis. In cell cycle regulation FOXOs transcribe p27kip1 (CDKN1B), which is a cell cycle kinase inhibitor (CKI) located downstream of FOXO. The transcribed p27kip1 is then able to induce cell cycle arrest at the G0/G1 phase, G2 phase, or result in complete senescence. These inhibitory effects are accomplished by inhibiting Cyclin/CDK complexes that participate in cell cycle progression, which is one of the disordered processes found to exist in cancer cells. Similarly, the loss of FOXOs functioning has been found to result in enhanced tumorigenesis in mice. (35) Additionally, FOXOs are able to induce apoptosis by inducing the expression of pro-apoptotic genes and anoikis by inducing extracellular matrix detachment. (36)

Dysregulation of FOXOs lead to multiple hallmarks of cancer formation. In addition to resisting cell death and sustained cellular proliferative signaling, some of these include cellular energetics, replicative immortality, genome instability and mutation. (37) In the cancer-immunity cycle, FOXOs have been found to regulate anti-cancer immunity in the tissues of healthy individuals. Notably, they promoted B cell differentiation as well as generating naïve CD4+ and memory CD8+ cells. However, when FOXOs are dysfunctional, additional hallmarks of cancer can be seen such as immune destruction and tumor-promoting inflammation. (38) FOXOs can also repress VEGF expression by directly binding to the VEGF-A promoter. Additionally, it was shown in a recent study that increased levels of the inactive phosphorylated form of FOXO family members were
correlated with increased levels of molecules required for angiogenesis. (39) Additionally, resistance to oxidative stress response is regulated by FOXO, which has been coupled to longevity. However, the dysregulation of FOXOs leads to a loss of this resistance and an increase in cellular damage. (40)

FOXO3 is a protein coding gene that is located on chromosome 6 in humans, namely at 6q21. This gene is explicitly responsible for encoding the transcription factor forkhead box O-3 (FOXO3). Since FOXO3 is part of the FOXO family of transcription factors, it contains the characteristic ‘Forkhead-box’ DNA-binding domains. Additionally, FOXO3 recognizes and binds to the 5’-TTGTTTAC-3’ DNA sequence in humans. Due to the fact that all four members of the FOXO family of transcription factors bind to this specific DNA sequence, it is sometimes referred to as being a redundant act of DNA recognition.

While studying the possible roles of FOXO3 in humans, recent evidence has come to show that this specific gene is associated with longevity. Initial studies began by using small nematodes called Caenorhabditis Elegans. They found that over time the expression levels of FOXO3 would decrease in these nematodes and noticeable signs of aging were present. (41) Further studies were conducted on C. Elegans to determine that FOXO3 proteins were present at increased levels among C. Elegans that lived almost twice as long as their counterparts which had low levels of FOXO3 expression. (42) Taking these findings into account, researchers were curious to see what additional roles FOXO3 had in humans. One study found that human longevity has a strong familial component and that the FOXO3 gene is a primary factor in having a longer lifespan. (43) Additional studies in humans agree on the notion of FOXO3 expression levels being a crucial element
responsible for increasing human lifespan and decreasing old-age-related ailments. (44) Moreover, variations of FOXO3 have been found to exist in certain populations around the world. In Japan, three different single-nucleotide polymorphisms (SNPs) were found among men that lived to be over 100 years of age. Moreover, men who were of Japanese ancestry and had these same FOXO3 variations were found to also have an increased life expectancy. (43) Based on these findings, populations in Germany and France were subsequently studied. Both populations of centenarians were found to have increased levels of FOXO3 gene expression. (45) Taken together, these findings illustrate the global importance of FOXO3 in multiple aspects of human health and prolonged survival.

In the context of cancer, FOXO3 is considered to be a tumor suppressor gene that regulates processes such as DNA damage repair, apoptosis, cell cycle arrest, as well as protection from oxidative stress. (46) Additionally, recent studies have suggested that FOXO3 might participate as a co-regulator of genes involved in colorectal cancer metastasis. One such study of colorectal cancer metastasis compared the level of FOXO3 expression that was seen in patients with metastasis and in patients who did not have metastasis, after having been diagnosed with colorectal cancer. Their results showed that there was reduced expression of FOXO3 in patients with stage I/II (node negative) colorectal cancer that subsequently developed metastasis. However, this lowered expression level of FOXO3 was not seen in patients that remained metastasis free. (47) Similarly to these findings, studies that compared FOXO3 levels from primary colon cancer tumors and corresponding liver metastases found that FOXO3 levels were drastically lower in the metastatic liver tumors. This finding reinforces the notion of
tumorigenesis and metastasis requiring lowered FOXO3 levels. (48) FOXO3 also has an important role in the regulation of intestinal inflammation both in vitro and in vivo. (49)

**Apoptosis:** Nuclear FOXO3 activates the transcription of several genes that encode pro-apoptotic proteins. Induction of the Bcl2 family of mitochondrial targeting proteins, increasing the expression of CDK inhibitors, triggering the expression of Fas ligand, and TNF-related apoptosis-inducing ligand. (50) However, activated AKT promotes cell survival by phosphorylating nuclear FOXO3 on several serine/threonine residues. This phosphorylation allows for phosphoserine-binding protein 14-3-3 to bind FOXO3 and export it from the nucleus into the cytosol. Once in the cytosol, inactive phosphorylated FOXO3 is unable to induce expression of these pro-apoptotic genes.

**Cell cycle regulation:** When FOXO3 is suppressed by activated AKT, it leads to impaired expression of the cell cycle inhibitor p27kip1. Thus, dysfunction of FOXO3 cell cycle regulation is an important mechanism that is involved in stimulating the proliferation of normal and transformed colonic epithelial cells. (51)

Oxidative stress and increased levels of reactive oxygen species (ROS) influence the translocation of FOXO3 into the nucleus via two main mechanisms. In the first mechanism, JNK can either directly phosphorylate protein 14-3-3 to prevent it from inhibiting FOXO3 or JNK can phosphorylate IRS1/2 and thereby indirectly prevent GFR-mediated inhibition of FOXO3. Regardless of whether JNK directly or indirectly prevents FOXO3 inhibition, the result is ultimately the same in that FOXO3 is able to translocate into the nucleus. (52) The second mechanism of FOXO3 translocation is accomplished by FOXO3 forming disulfide bridges between their cysteine residues and nuclear importers, such as TNPO1, IPO7, or IPO8. Once FOXO3 is inside of the nucleus, it induces
transcription of multiple antioxidant genes. (53, 54, 55) Similarly, when cells encounter stress caused by nutrient deficiency and decreased ATP levels, AMP kinase (AMPK) is activated. AMPK directly phosphorylates three residues on FOXO3, which include Ser143, Ser588, and Ser626. This mechanism leads to the translocation of FOXO3 into the nucleus where it stimulates target genes that are involved in cellular stress resistance. (56)

Drug resistance in colon cancer cells is an associated consequence of FOXO3 inactivation. Low levels of FOXO3 in colon cancer have recently been shown to result in chemotherapeutic resistance to cisplatin. (57) The changes in cell signaling that is caused by decreased levels of FOXO3 is thought to be the contributing factor of chemoresistance and poor prognosis in some colon cancer patients. Specifically, low levels of FOXO3 in colorectal cancer cells contributes to cetuximab resistance. (58)

Lipid Droplets (LDs) are intracellular lipid storing organelles that are primarily found in adipose tissue, and to a lesser extent in non-adipose tissue such as the colon. Although these novel organelles were once classified as inert lipid globules, recent studies have demonstrated the significant roles they have in various intracellular trafficking pathways correlated to disease development. Lipid droplets mainly consist of neutral lipids (triglycerides/triacylglycerols, sterols, and sterol esters) surrounded by a special monolayer of phospholipids that act as a natural surfactant. (59-60)

Structure: The appearance and size of lipid droplets can vary, to a certain extent, among organisms and cellular location. Diversity in the size of LDs can range between sub-μm to 200 μm in diameter. Regardless of these size differences, the structure and organization of lipid droplets is highly conserved. (61) Recently, LDs have been found to have the ability to establish functional contact sites with several other organelles via surface
membrane interactions. Although the surface portion of lipid droplets are exceedingly similar to the surface of the endoplasmic reticulum, investigators wondered how this structural similarity enabled lipid droplets to bind and interact with proteins that possess contrasting molecular structures. Up until this point, the proteins that were able to interact with LDs, were previously only known to interact with the ER and/or the Golgi apparatus. One commonality found among these proteins is an amphipathic helix (AH) region. (62)

Attempts to further explain this phenomenon have been made in more recent years. More importantly, LD membranes possess physiochemical properties that allow for their interaction with proteins. Such properties include electrostatic forces, surface tension, and curvature of the membrane as the primary regulatory physiochemical properties. Similarly, these same physiochemical properties are suggested as being a primary regulatory factor involved in the process of LD budding from the ER during lipid biogenesis. (63) How the interfacial structure of LDs pertains to its functional regulation has been difficult to determine, primarily because of experimental and computational limitations. In attempts to solve this dilemma, investigators developed a combination of computational approaches that were optimized to study oil-water interfaces. (64) A recent study employed this combinational approach in developing LD surface simulations. Their findings suggest that underlying metabolic processes involved in disease development are the result of unique structure-function relationships seen with characteristic LD features. (65)

**Functions:** Lipid droplets are involved in the production of inflammatory mediators as well as the process of lipid metabolism. (66) LDs are also sites of cellular regulatory events, energy homeostasis, fat storage, mobilization control, and direct interactions with other organelles such as the mitochondria and peroxisomes, in addition to the ER. The
fundamental processes of lipid synthesis along with the storing of neutral lipids in LDs is
accomplished in eukaryotic cells. A protective role is exerted by scavenging free fatty acids
that would otherwise result in lipotoxicity and eventual lipoapoptosis. In part, it has been
suggested that this protective role is accomplished via LD interactions that occur directly
with the mitochondria. Once free fatty acids have been released from LDs, they can be
channeled into energy production via β oxidation. Fatty Acid Oxidation (β-oxidation) is a
catabolic pathway of fats in which 3 fatty acids are converted to Acetyl CoA. Due to the
fact that this process utilizes oxygen, it is referred to as an oxidation reaction. Fats are
stored in the human body in various forms but the major storage form is triglycerides.
Triglycerides consist of a single glycerol molecule that is attached to three fatty acid
molecules. The structure of fatty acid consists of a long chain of carbon and hydrogen
atoms. In β-oxidation, the bond between the α and β carbons is broken, coinciding with
why this is called β-oxidation. Adipose tissue consists of triglycerides, which contain fatty
acids and glycerol molecules. The fatty acids are then distributed throughout the body, after
entering the circulation, and eventually reaching target cells which are able to metabolize
fatty acids. Free fatty acids have a net negative charge and are unable to cross membranes
without the use of transporters. After entering the cytoplasm of these target cells, the fatty
acids pass through the inner and outer mitochondrial membranes to enter the matrix where
they proceed to be metabolized via oxidation. (67, 68, 69)

Interactions between peroxisomes and LDs have been demonstrated in
Saccharomyces Cerevisiae yeast. Peroxisome extensions, known as pexopodia, extend into
the LD core. Defects in peroxisome function led to changes in the morphology of LDs.
Additionally, electron-dense regions in the LD core became curled and tangled. These
findings suggest that lipid biogenesis is the result of a coordinated effort between multiple organelles. (70)

**Regulation:** The ER compartment and LD compartment might stay in contact with each other throughout the life cycle of lipid droplets. (71) Perilipin/PLIN1 is a phosphoprotein in adipocytes that has an important role in regulating both basal and stimulated lipolysis. The discovery of PLIN1 was the first time that researchers could demonstrate that LDs were involved in more than just storage and a passive role in energy homeostasis. (72) Expansion of this finding led to the realization that PLIN1 was not the only LD-associated protein that enabled regulatory functions of LDs. Currently, an entire perilipin-like protein family has been established. Namely, these include perilipin/PLIN1, ADRP/Adipophilin/PLIN2, and Tip47/PLIN3. All members of this protein family contain an evolutionarily conserved N-terminal domain that consists of approximately 100 amino acids and has been termed the PAT-domain. Ongoing studies are attempting to determine the extent to which PAT-domain proteins correlate LDs to regulatory cellular processes. Thus far, findings have confirmed that PAT-domain proteins are a primary mechanism in which lipid droplets are able to participate in the regulation of lipid metabolism. (73-74)

**Triglycerides (aka triacylglycerols)** are a major type of neutral lipid found in the body. They are produced endogenously by the liver or can be absorbed from food in the small intestine. Structurally, TGs consist of three fatty acids attached to a molecule of glycerol by ester bonds. Although the body can store potential energy in three macromolecule forms, namely glycogen, triglycerides, and proteins, TGs are able to sustain our energy needs for longer periods of time. This is made possible by two properties that make TGs highly compact stores of cellular energy. First, TGs are highly reduced
molecules which enables them to undergo many cycles of oxidation and thus form a large number of energy molecules. Second, they are anhydrous which enables our cells to store more kJ/gram of energy in a more condensed format. In humans, TGs are stored in the cytoplasm of adipose cells in the form of fat globules until they are needed for ATP production. Although TGs are essential macromolecules, their excess accumulation in adipocytes leads to obesity and in non-adipose tissues leads to organ dysfunction. (75, 76, 77)

Triglyceride Synthesis (aka Lipogenesis) is the synthesis of fatty acids, and eventually triglycerides (TG)/triacylglycerols (TAG). The amount of glycogen that our bodies can store, in places like the liver and in muscle, will eventually reach a saturation point. Once this saturation point has been reached, de novo lipogenesis is initiated and fat storage ensues. The storage of triglycerides occurs predominantly in adipose tissue. In a two-step process, fatty acid thiokinase activates fatty acids (FA) by catalyzing the formation of a thioester bond between the carboxyl group of the fatty acid and the sulfur group of coenzyme A. In its activated form, namely Acyl-CoA, fatty acids can then be added to the carbons of the glycerol-3-phosphate backbone. An acyl group of the first Acyl-CoA, which is usually saturated, is bound to the first carbon of glycerol-3-phosphate by Acyl-Transferase. Next, an acyl group from a second Acyl-CoA, which is usually unsaturated, will be bound to the second carbon of glycerol-3-phosphate by Acyl-Transferase. These steps result in the formation of phosphatidate (DAG-3-phosphate) which is further converted into diacylglycerol (DAG). Once again, Acyl-Transferase catalyzes the addition of a third acyl group from an Acyl-CoA, resulting in the final form of triglycerides/triacylglycerols. Acyl-Transferase (DGATs) enzymes are essential to these
steps of triglyceride synthesis and will be discussed in further detail within the upcoming sections. (78-82)

**LD Formation:** During LD biogenesis, the final phase of triglyceride production uses both DGAT1 and DGAT2. After the synthesis of triglycerides occurs, they travel to the endoplasmic reticulum (ER) which contains both DGAT1 and DGAT2 embedded on the surface. While in the endoplasmic reticulum, DGAT1 and DGAT2 enzymes function to initiate the formation of lipid droplets. Once lipid droplets are formed, they detach from the endoplasmic reticulum and enter the cytosol. While in the cytosol, lipid droplets continue to grow and mature primarily due to the presence of DGAT2 on the surface of lipid droplets. In comparison, both DGAT1 and DGAT2 are present in the endoplasmic reticulum. However, only DGAT2 is present on the surface of lipid droplets themselves. (83) Several models have been suggested, that aim to identify the details of how newly synthesized triglycerides are deposited into lipid droplets specifically. (84)

**Colon Cancer Connection:** In the context of cancer, upregulated lipogenesis has been found to be a common phenotype seen among numerous human carcinomas. Moreover, it has been associated with poor prognosis in breast, prostate, and colon cancer. (85, 86) Increased levels of lipid droplets have been found in both human colon cancer tissue and animal models. Studies have shown findings that suggest increased lipid droplet formation is an important factor in human colon cancer proliferation and progression. In non-adipose tissue, such as the colon, a link between loss of FOXO3 function and increased LD synthesis was seen in colon cancer patients. High-fat-diet (HFD) mediated obesity bolsters colon cancer progression and increases lipid droplet accumulation in non-adipose tissues, such as the colon, as well as in adipose tissue itself. (87)
Diacylglycerol O-Acyltransferase 1 (DGAT1) belongs to the family of membrane-bound O-acyltransferases (MBOAT) and was originally discovered as a result of its homology to acyl CoA:cholesterol acyltransferase (ACAT)-1 and -2, which is responsible for catalysis of cholesterol ester biosynthesis. In humans, the DGAT1 gene is located on chromosome 8 (8q24.3). Structurally, this 55 kDa integral membrane protein has three transmembrane domains and can exist in both dimer or tetramer formations. The cytosolic facing N-terminus of DGAT1 is responsible for tetramer formation, in addition to regulating DGAT activity, while the C-terminus is located within the endoplasmic reticulum lumen. Moreover, the C-terminus of DGAT1 contains highly conserved histidine (His-426) which is part of the active site and is crucial for the overall function of DGAT1. (88, 89, 90)

**Location:** Greater amounts of DGAT1 have been found in locations such as adipose tissue, heart, mammary gland, thymus, spleen and the small intestine. Notably, DGAT1 is highly expressed in the intestine. One commonality that these locations possess, is their ability to produce copious amounts of triglycerides. (91) A study that was conducted on the effects of lost DGAT1 function, compared the outcomes of chemically inhibited DGAT1 and genetic deficiency of DGAT1. Under both circumstances, genes involved in lipid uptake and oxidation were found to be suppressed. Thus, DGAT1 inhibition resulted in genetic suppression that ultimately led to a protective role against lipotoxicity. (92-93)

**Function:** The DGAT1 enzyme is essential for TG biosynthesis in eukaryotic cells. With the use of competition assays, DGAT1 was found to display a preference for monounsaturated substrates, such as oleoyl-CoA, when compared to saturated substrates. These findings suggest that DGAT1 might have a more active role in specifically
incorporating monounsaturated fatty acids into triglycerides during TG synthesis. (94) To further investigate this possibility, researchers used genetically modified mice. Mice that were deficient in DGAT2 were born much smaller and often died after only a few hours. However, mice that were deficient in DGAT1 survived and were much more physically lean. Even when put on a high-fat-diet, DGAT1 deficient mice were resistant to HFD-mediated obesity. Moreover, triglyceride levels in DGAT1 deficient mice were substantially lower in non-adipose tissues. These findings strongly suggest that DGAT1 levels have a proportional relationship to fat storing capacity both in adipose tissues and non-adipose tissues, regardless of diet. (95-96) Investigators wanted to know the underlying mechanism behind how these mice were able to resist obesity. The initial presumption made, was that fat malabsorption might be the reasoning behind these results. However, one study found that DGAT1 deficient mice had slower gastric emptying and a decreased rate of TG absorption from the GIT. Defective fat absorption was not found to have any association with obesity resistance in such studies. Therefore, it is suggested that the role of DGAT1 includes an accelerated assimilation of triglycerides. (97-98) When looked at in unison, these findings suggest that altering the levels of DGATs enzymes, and more specifically of DGAT1, could be implemented as a possible therapeutic target in obesity-related colon cancer.

Connection To Colon Cancer: Preliminary data has shown that there are increased levels of DGAT1 and DGAT2 enzymes in mouse colonic tissue. More specifically, there were increased levels of DGATs found in the walls lining the proliferative crypts. (99)

**Diacylglycerol O-Acyltransferase 2 (DGAT2)** was originally discovered by purifying lipid fractions from a fungus known as Mortierella Ramanniana. Subsequently,
there are several homologous mammalian sequences that have been identified and collectively grouped under the DGAT2 gene family. In humans, the DGAT2 gene is located on chromosome 11 (11q13.3). Structurally, this 44kDa integral membrane protein is primarily found in the endoplasmic reticulum and has two transmembrane domains with both the N- and C-termini facing the cytosol. Unlike DGAT1, there are two highly conserved sequences that are necessary for the functional activity of DGAT2. One of these sequences (His-Pro-His-Gly) is only present in members of the DGAT2 family and is part of the active site. The other unique sequence that is found in DGAT2 (FLXLXXXn) is a neutral lipid binding domain that has been found to be present among other proteins, primarily in proteins that are involved in lipid metabolism. (100, 94)

**Location:** DGAT2 usually resides on the endoplasmic reticulum surface. However, induction of TG synthesis with oleic acid leads to the presence of DGAT2 close to the surface of lipid droplet membranes. This superficial orientation on lipid droplets permits the DGAT2 enzyme to co-localize with the mitochondria. (83) Further investigation of this process, with the use of biochemical fractionation and immunofluorescence microscopy, has revealed that DGAT2 is specifically concentrated in the mitochondrial-associated-membrane (MAM) region of the endoplasmic reticulum. Additionally, DGAT2 interacts with the mitochondria via its N-terminal amino acid sequence. Two significant sequences were discovered during one such study, each with different properties. Although both amino acid sequences act as targeting signals for mitochondrial-DGAT2 interactions, the 67 N-terminal amino acids of DGAT2 are not conserved and thus have varying functions between family members of this enzyme. On the other hand, a murine mitochondrial
targeting sequence does possess a conserved sequence that is located between amino acids 61 to 66. (101)

**Function:** The DGAT2 enzyme is primarily expressed in adipose tissue and the liver. It is also present in intestinal cells, similar to DGAT1, but to a lesser extent. In addition to its broad role as a lipogenic enzyme, DGAT2 is specifically involved in catalyzing the final step of triacylglycerol (TAG) synthesis in eukaryotic cells. In comparison to DGAT1, data suggests that DGAT2 possesses a more potent enzymatic activity as well as a higher affinity for the substrates that it interacts with. Additionally, DGAT2 has not been found to display a preference for monounsaturated substrates over saturated substrates. Although saturation preferences were not found to be relevant to DGAT2, there was enhanced enzymatic activity noted with regard to medium-chain fatty acyl-CoAs when compared to short or long-chain fatty acyl moieties. (94, 100, 102)

**Connection To Colon Cancer:** A genome-wide study has linked the chromosomal region that DGAT2 is located on, namely 11q13, to obesity. (95) As obesity has become a global health concern and has been determined to be a colon cancer risk factor, this could be a significant element during future studies of DGAT2 in CRC. (103)

**Tumor-Necrosis-Factor Alpha (TNFα)** is a cytokine that had initially gained recognition for its principle role in causing hemorrhagic necrosis in tumors. However, recent studies have investigated the possible role that TNFα has in mediating cancer-related inflammation. Although a connection between host inflammation and cancer has been suspected for the greater part of the 20th century, recent epidemiological studies have shown that chronic inflammation does in fact influence the formation of multiple cancer types. While keeping in mind that TNFα is a primary inflammatory cytokine, researchers
were interested in finding out how the pathways and mediators participate in cancer-related inflammation. Notably there are two pathways, an intrinsic and an extrinsic, that have been identified. The intrinsic pathway implements genetic factors that cause cancer development, while noting that such genetic events lead to the formation of an inflammatory microenvironment that is a key underlying factor in cancer development. Similarly, the extrinsic pathway implements inflammatory conditions, such as inflammatory bowel disease, as being a vital element in the facilitation of colon cancer development. Further strengthening these findings is that colitis-associated cancer arises in patients with ulcerative colitis, in which the colonic mucosa is chronically inflamed. An interconnection between these two pathways was found to include transcription factors and primary inflammatory cytokines, such as TNFα. (104-105) Adipose tissue regulates energy balance and lipid metabolism via the release of adipokines such as leptin, adiponectin, resistin, and tumor necrosis factor-α (TNFα).

In patients with inflammatory bowel disease, TNFα has been found to play an essential role in maintaining intestinal inflammation which correlates to an increased risk of developing colon cancer. Upon TNFα binding to the cell membrane receptors TNFR1 and TNFR2, inhibitory kappaB (IkB) kinase complex (IKK) is activated. Activated IKK results in the release and translocation of NFκB into the nucleus where it functions as a regulator of genes that are implemented in inflammation, cell survival, and proliferation. (106) One study built on the idea that FOXO3 somehow plays a role in the intestinal inflammation process that predisposes an increased risk of developing colon cancer. In unison with TNFα being a major contributor to intestinal inflammation, they found that TNFα inactivates FOXO3 through the IKK and/or PI3K pathways. In both of these
pathways, TNFα caused increased phosphorylation of FOXO3 which lead to its translocation from the nucleus to the cytosol where it remained inactive. Although IKK phosphorylates FOXO3 at Ser644 and PI3K phosphorylates FOXO3 at Thr32, the increased phosphorylation and subsequent deactivation of FOXO3 was seen to the same extent in both pathways. (107-108)

**PI3k/PKB/AKT pathway:** The activation of AKT is strongly associated with the proliferative and pro-survival activities of PI3K. AKT is also known as protein kinase B that functions as a serine and threonine kinase, leading to the phosphorylation of serine and threonine residues on target proteins. There are three isoforms of AKT: AKT1 (PKBα), AKT2 (PKBβ), and AKT3 (PKBγ). AKT is regulated by growth signals and it is involved in various disease states, including cancer, insulin resistance, type 2 diabetes, cardiovascular diseases, and autoimmune diseases. Activation of AKT begins at the cell membrane with the activation of either a Receptor Tyrosine Kinase (RTK) or G-Protein Coupled Receptor. Although these are two different types of receptors, both of them lead to the activation of AKT. Once either of these receptors are activated, a Phosphatidylinositol-3-Kinase (PI3K) heterodimer is recruited to the cell membrane and subsequently phosphorylated to become active. Activated PI3K is then able to phosphorylate phosphatidylinositol 4,5-bisphosphate (PIP2) that is present on the intracellular surface of the cell membrane and thereby converts it into phosphatidylinositol 3,4,5-triphosphate (PIP3), which recruits 3-phosphoinositide-dependent protein kinase 1 (PDK1) to the cell membrane. PDK1 is able to directly phosphorylate the Threonine 308 residue (Thr308) on AKT as well as indirectly activate Mammalian Target of Rapamycin Complex 2 (mTORC2) which will phosphorylate the Serine 473 residue (Ser473) on AKT.
In-vitro studies have shown that the phosphorylation of both Thr308 and Ser473 sites led to increased AKT activity. Moreover, the phosphorylation of Thr308 is essential for AKT activation while the phosphorylation of Ser473 is essential for the ability of AKT to interact with downstream targets, specifically forkhead transcription factors. (100) Once AKT is activated, it functions in the regulation of multiple cellular processes. These include increasing glycolysis by increasing the uptake and utilization of glucose, increasing translation and protein synthesis by mTORC1 activation, increases glycogen synthesis by activating glycogen synthase, increasing fatty acid synthesis by activating ATP Citrate Lyase, prevention of apoptosis and autophagy by phosphorylating BAD, procaspase-9 and FOXO forkhead transcription factors. (109-114)

pAKT Inactivates FOXO and p27kip1: FOXO proteins normally inhibit cell survival and proliferation but once FOXO proteins are phosphorylated by activated AKT that has relocated to the nucleus, the pFOXO is rendered inactive and cannot fulfill these regulatory functions. By this interaction, AKT actually increases cell survival and proliferation, both of which are key hallmarks of cancer cells during carcinogenesis. (115) Thus, the PI3K-PKB/AKT pathway is involved in negatively regulating transcriptional activity of the FOXO1, FOXO3, and FOXO4 members of the FOXO transcription factor family. Furthermore, pAKT phosphorylates anti-proliferative regulators that include p21Cip1 (CDKN1A) and p27Kip1 (CDKN1B) in the cytoplasm. The resulting affect is that these anti-proliferative regulators are unable to accomplish their cell cycle arresting functions.

Turning Off The Pathway: Increased activity of the PI3K-PKB/Akt pathway has been associated with resistance to chemotherapeutic drugs in various types of cancers and
inhibiting this pathway has been shown to reverse this resistance. Turning off the PI3K-PKB/Akt pathway can be accomplished by the cell itself at various points. At the beginning of the pathway, PTEN is a phosphatase that downregulates PIP3 by converting it back into PIP2 which results in PDK1 not being recruited and activated. Another way that AKT signaling can be turned off is through a phosphatase called PHLPP that is responsible for de-phosphorylating AKT at its Ser473 residue. Lastly, protein phosphatase 2A (PP2A) is a phosphorylase that de-phosphorylates AKT at its Thr308 residue. (116) In the past decade, pharmaceutical inhibition of the PI3K-PKB/Akt pathway has been investigated. As mTOR is a primary element of regulation in this pathway, the pharmaceutical inhibition of mTOR signaling has provided evidence that suggests a potent anti-neoplastic result. Since inhibiting this key pathway regulator has resulted in potent anti-neoplastic effects, it raises questions regarding how pharmaceutical measures to prevent cancer development could be taken for AKT targets such as FOXO3. (117)
PART II. PROJECT OBJECTIVES

PROBLEM STATEMENT

Human colon cancer progression is associated with loss of FOXO3 tumor suppressor function. Furthermore, previous studies have shown that a loss of FOXO3 activity is linked to colon cancer proliferation, progression, and metastasis. In parallel to these findings, increased lipid droplet accumulation in non-adipose tissue, such as the colon, has recently gained interest. Although the underlying mechanism of increased lipid droplet accumulation in colon cancer patients is not fully understood, studies suggest a mechanism between loss of FOXO3 function and the noticeably increased levels of lipid droplets. As these organelles are a primary source of energy storage, they have a critical role in providing proliferating cancer cells with the energy that they require.

In human colon cancer cells, increased levels of Acyltransferase DGATs enzymes have been noted within previous studies conducted in our lab. Moreover, increased levels of both DGAT1 and DGAT2 were seen. These enzymes are essential during the final stage of triglyceride synthesis and lipid droplet formation. Therefore, their increased levels in both human and mouse models suggests that they could potentially be a therapeutic target or biomarker in colon cancer. For this reason, further investigation of whether FOXO3 function could be regained in colon cancer cells by pharmacological inhibition of DGAT1 and DGAT2 enzymes after induction of lipid droplet formation.
SPECIFIC AIMS

1. In human colon cancer cells, does blockade of DGAT1 and DGAT2 prevent Oleic Acid (OA)-mediated loss of FOXO3?

2. In human colon cancer cells, does blockade of DGAT1 and DGAT2 attenuate PI3K-mediated loss of FOXO3?

3. In human colon cancer cells, does blockade of DGAT1 and DGAT2 prevent Tumor Necrosis Factor α (TNFα)-mediated loss of FOXO3?
PART III. EXPERIMENTAL PROCEDURES

MATERIALS AND METHODS

Cell Culture: Media and Growth Conditions

Human colon cancer HT29 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). These intestinal epithelial cells were cultured and propagated in complete McCoy's 5A media (Sigma-Aldrich, St Louis, MO, USA) containing 10% fetal bovine serum (Gibco, Carlsbad, CA, USA). Each passage included the following steps: aspiration of McCoy's 5A media, add 1mL of Trypsin and leave at room temperature for 30 seconds, aspirate current Trypsin and add 2mL of new Trypsin followed by a 2-4 minute long time period in an incubator at 37°C. After this step, 8mL of fresh McCoy’s 5A media was added to the existing 2mL of trypsin, totaling 10mL in the plate. Lastly, 1mL from this plate was transferred to a new cell culture plate that contained 9mL of fresh McCoy’s 5A media. Prior to treatments, HT29 cells were plated on 6-well plates. When the monolayers reached confluency of ~70% cells were serum-starved overnight before the experimental procedures were performed. Cells were grown under mycoplasma-free conditions at 37°C in humidified 5% CO₂.

Treatments and Preparation

Treatments: Oleic Acid--LD accumulation was induced with 1 mg/ml of oleic acid (OA) (Sigma) over a 1 h time period. TNFα--HT29 cells were treated with 10 ng/ml of human recombinant TNFα (R&D Systems, Minneapolis, MN, USA) over designated time periods of 1 h and 2 h.
Inhibitors/ Blocking Agents: For blockade of DGAT1 and DGAT2 activity, the following pharmacological inhibitors were employed: DGAT1 inhibitor A922500 (Sigma, 50 μM); DGAT2 inhibitor, PF06424439 (Sigma 50 μM).

Protein Extraction and Processing

After treatment, the HT29 monolayers were washed with PBS (Gibco). Then total and fractionated protein was extracted by applying cell lysis buffer (Cell Signaling, Danvers, MA, USA), containing a protease inhibitor cocktail (Sigma). 10x stock concentration RIPA buffer was diluted to a 1x concentration with diH2O. While chilling the 1x RIPA buffer on ice, 200x stock concentration PMSF was diluted to a 1x concentration. The 1x PMSF was then added to the 1x RIPA buffer, forming a 1mM final concentration, just prior to use. 200µl of RIPA-PMSF was then added to each well of the 6-well plates and were left on ice for five minutes before scraping each well. After thoroughly scraping each well, the RIPA-PMSF-Cell mixture was collected into microcentrifuge tubes and vortexed at 14,000 RPM for 10 minutes in a 4°C cold-room to separate the supernatant and pellet. The supernatant was then collected and transferred into new microcentrifuge tubes, leaving behind the pellet. The pellet is the whole cell lysate and was stored separately in a −80°C freezer and the supernatant is the stock cytosolic fraction that was used for further processing.

Protein concentrations were determined using the Bradford Protein Assay method. A working solution was prepared by mixing Reagent A and Reagent B in a dilution scheme 50:1 ratio of A:B respectively. Using a 96-well plate, 5-10µl of each unknown sample was run in duplicate with 200µl of homogeneous working solution. For preparing the standard curve with BSA known protein samples, 25µl of each standard was run in duplicate with
200µl of homogeneous working solution. After five minutes of mixing on a rocker at room temperature, incubation at 37°C took place for ten minutes. The plate was then cooled to room temperature and read on a spectrophotometer at 562 nM. Concentrations obtained from the spectrophotometer were then plotted and used to calculate the loading amounts for each individual sample. The protein substrates were diluted in a 2:1 ratio with 2x Laemmli Sample Buffer (50mM Tris, pH 6.8, 2% SDS, 100 mM dithiothreitol, 0.2% bromophenol blue, 20% glycerol) containing the added element of 2-mercaptoethanol, according to manufacturer instructions, and boiled for five minutes. After enough time had elapsed to ensure proper cooling, equal amounts of protein were then loaded onto 10% acrylamide gels, using loading amounts that were determined based on the Bradford Protein Assay analysis. Remaining protein samples were stored at −80°C until further processing.

**SDS-PAGE and Western Blot**

As a marker, Chameleon Duo Pre-Stained Protein Ladder (LI-COR) was used. Following this step, equal amounts of protein were loaded onto SDS-PAGE (Bio-Rad) and separated by running the gel at 120V for approx. 2-3 hrs. The proteins were subsequently transferred to nitrocellulose membranes (Bio-Rad Transblot-Turbo) and incubated in blocking buffer (5% BSA in 0.1% TBST) for one hour at room temperature.

**Detection and Probing**

After blocking for one hour at room temperature with blocking buffer, the immunoblots were probed with 5% BSA-TBS solution containing specific primary antibodies (Cell Signaling Technology, Danvers, MA) to perform detection. An amount of 5mL of diluted primary antibody was added to the nitrocellulose membrane paper, ensuring
that even coverage occurred. Incubation with each primary antibody was done overnight in a 4\(^\circ\) cold-room with continuous agitation by use of a rocker. Following this step, membranes were washed in 0.1% TBST three times, for five minutes each time. (Table 2)

IR-tagged Secondary Antibodies from Li-Cor Biosciences (Lincoln, NE) were used to perform detection after overnight incubations with each primary antibody. An amount of 5mL of diluted secondary antibody in 5% BSA-TBS solution was added to the nitrocellulose membrane paper, once again ensuring that even coverage occurred. For the duration of two hours, at room temperature, probing with secondary antibodies occurred while maintaining continuous agitation with use of a rocker. Following this step, membranes were washed in 0.1% TBST three times, five minutes each time. After washings were completed, blots were analyzed by the Odyssey Infrared Imaging System (LI-COR Biosciences). (Table 3)

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**Table 2. Primary Antibodies Used**

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**Table 3. Secondary Antibodies Used**
**Statistical Analysis**

All data are means ± S.E. for a series of experiments. Statistical analysis was performed by Student’s unpaired t-test or one-way analysis of variance and Student-Newman-Keuls post-test by using Graph Pad Instat 3 software (Graphpad Software, San Diego, CA). A p value <0.5 was considered to be significant.

**Immunofluorescent Staining**

For immunofluorescent staining, HT29 cells were grown on coverslips. The coverslips were initially placed in 70% EtOH for about 30min (10cm dish) before being removed from the dish and transferred to 24well plates containing 70% EtOH. Incubation lasted for 30 minutes, after which time the EtOH was aspirated and each coverslip was washed with PBS. HT29 cells were then seeded and given one day to allow for attachment to occur, prior to starting the desired treatment. After treatment, the media was aspirated and 500µl of 4% formaldehyde solution was added to each well. They were then left for 10 minutes at room temperature to allow for fixation to ensue. The fixed cells were washed 2-5 times with PBS and permeabilized with 0.15% Triton-X in PBS. Washing with 500µl of PBS three times was followed by blocking in 2.5% bovine serum albumin for 1 h at room temperature. The fixed cells were then incubated with a primary FOXO3 antibody (Upstate, Billerica, MA, USA) in 2.5% BSA for 1 h at room temperature on parafilm. Following this 1 h incubation, the coverslips were transferred back into the well plate and washed three times with PBS. Next, incubation with a secondary antibody (Alexa Fluor 488 Molecular Probes, Eugene, OR, USA) in 2.5% BSA for 1 h at room temperature on parafilm, while avoiding any exposure to direct light. Following this 1 h incubation, the coverslips were once again transferred back into
the well plate and washed three times with PBS. Lastly, 300ul per well of 1 ug/mL DAPI stain in PBS was used to stain and detect the cell nuclei. While ensuring that the coverslips are protected from direct light exposure, they were incubated in DAPI for 5 minutes at room temperature. After this incubation was completed, the coverslips were washed 3 times with PBS before removing them and allowing excess PBS to evaporate off, while simultaneously ensuring that the coverslips don’t dry out too long. The coverslips with cells were then mounted onto microscope slides, using Mounting Solution antifade reagent, Molecular Probes. The results were assessed using a Nikon Opti-Photo microscope. The images were captured using a Spot RT-slider camera (Diagnostic Instruments, Sterling Heights, MI, USA) and managed by Image Pro software (Media Cybernetics, San Diego, CA, USA).

**Imaging Analysis**

**Coverslips**— The results were assessed using a Nikon Opti-Photo microscope. The images were captured using a Spot RT-slider camera (Diagnostic Instruments, Sterling Heights, MI, USA) and managed by Image Pro software (Media Cybernetics, San Diego, CA, USA).

**Immunoblots**— Following incubation with secondary antibodies, membranes were washed three times for 5 minutes per wash in TBS containing 0.1% Tween, and blots were analyzed by the Odyssey Infrared Imaging System (LI-COR Biosciences). Data was normalized to actin and served as a loading control. Each experiment was conducted in triplicate and is represented by the blots present hereafter. Experiments were conducted in triplicate with representative blots shown. Densitometric analysis of the blots were performed using Image J Software (NIH).
PART IV. FINDINGS

RESULTS

DGAT1/2 Inhibition Protects Loss of FOXO3 After OA-mediated Increased LD Density

Previously, our lab has shown that OA mediates increased LD density, in part, through loss of FOXO3. Lipid droplets are important as they provide fuel energy for proliferating cancer cells and lipids for new membrane synthesis in dividing cells. Given our interest in better understanding this regulation, I sought to determine the role of DGAT1/2 in OA-mediated FOXO3 loss. For this reason, by utilizing pharmacological inhibition of DGAT1/2, I examined the resulting effect on FOXO3 following OA treatment. After stimulating increased lipid droplet density to occur with OA, there was a visible loss of FOXO3. Additionally, I observed FOXO3 function in cells that had been co-treated with OA/DGAT inhibitors compared to OA alone. My results show that in human colon cancer HT29 cells, pharmacological blockade of DGAT1/2 prevented OA-mediated loss of FOXO3 (degradation) as shown by western blot (Figure 1) as well as by immunofluorescence staining (translocation from the nucleus to the cytosol) (Figure 2). Ultimately, my findings demonstrate that increased lipid droplet accumulation does indeed cause FOXO3 translocation from the nucleus to the cytosol, leading to its degradation. Moreover, inhibition of DGAT1 and DGAT2 enzymes result in a reversal of this process leading to the reinstatement of FOXO3 tumor suppressor and transcription factor function.
**DGAT1/2 Inhibition Protects Loss of FOXO3 After OA Treatment Through Inhibition of Upstream PI3K Signaling.**

In order to determine potential regulators of this feedback regulation network between lipid droplets and FOXO3 and DGAT inhibition, I assessed the functional activity of the PI3K pathway. PI3K is a kinase involved in multiple cellular functions including growth and metabolism, and it has previously been shown to lead to the inactivation of FOXO3. By inactivating FOXO3, active AKT functions to increase cell survival and proliferation, both of which are key hallmarks of cancer cells during carcinogenesis. Thus, the PI3K-PKB/AKT pathway is involved in negatively regulating transcriptional activity of FOXO3. After stimulating increased lipid droplet density to occur with OA, there was an increased level of phosphorylated Akt in human colon cancer cells. Since Akt is the direct target of PI3K, the increased levels of activated pAkt indicate an increased level of PI3K pathway activation. As stated in earlier sections, overly activated PI3K is an underlying reason for colon cancer progression, as well as progression in multiple other cancer types. Therefore, finding agents that may prevent PI3K activation and restore FOXO3 tumor suppressor function are of high interest. My results show that in human colon cancer cells, pharmacological blockade of DGAT1/2 prevented upstream activation of PI3K-Akt activity (Figure 3) as measured by reduced levels of phosphorylated-Akt status in cells co-treated with OA/DGAT inhibitors compared to OA alone. Thus, indicating that DGATs protective effect on FOXO3 status (Figure 1 and 2) is mediated, in part, through inhibition of PI3K signaling.
DGAT-Mediated Protection of FOXO3 Corresponds with Functional Downstream Recovery of p27kip1

Previous studies investigated the role of increased lipid droplet density, seen in human colon cancer, on the cell cycle. Their findings showed that when lipid droplet density is increased in human colon cancer cells, FOXO3 function is lost as well as decreased expression of the cell cycle inhibitor p27kip1. Furthermore, decreased expression levels of p27kip1 have been linked to increased proliferation and tumor growth capabilities within the context of colon cancer. To investigate whether p27kip1 function could be retrieved after DGAT1/2 inhibition, I compared p27kip1 expression levels before and after OA treatment either alone or co-administered with DGAT1/2 inhibitor treatments. After inducing lipid droplet density with OA, there was a visible loss of FOXO3 function and a subsequent decrease in the expression of p27kip1. Both of these findings were to be expected, however, I wanted to see if expression levels could be reestablished by using DGAT1/2 inhibitors. My findings showed that p27kip1 function remained at normal levels when DGAT1/2 inhibitors were used (Figure 4), indicating a functional role for DGAT inhibition in mediating FOXO3 downstream effects.

DGAT1/2 Inhibition Protects Loss of FOXO3 After TNF treatment through inhibition of PI3K-Akt activation

Additionally, our lab has also shown that the inflammatory cytokine TNF is a critical regulator of FOXO3 function, in which treatment of colon cancer cells with TNF leads to loss of FOXO3 function and can induce LDs. Given our interest in better understanding the role of DGAT function in the regulation of FOXO3 I utilized
pharmacological inhibition of DGAT1/2 and examined the resulting effect on FOXO3 following TNF treatment. After stimulating HT29 colon cancer cells with TNF for 1 and 2 hr, there was a visible increased level of phosphorylated FOXO3 correlating with an overall loss of FOXO3 (Figure 5). Additionally, I observed FOXO3 function was restored in cells that had been co-treated with TNF/DGAT inhibitors compared to TNF alone. This was shown by DGAT inhibition reducing levels of phosphor-FOXO3 and leading to recovery of overall levels of total FOXO3 (Figure 5). Furthermore, I found that DGAT inhibition were reducing levels of pAKT, which mechanistically provide reason for recovery of FOXO3 (Figure 6). Ultimately, my findings demonstrate that inflammatory TNF treatment of colon cancer cells leads to reduced FOXO3 function, in part, through transactivation of PI3K-Akt signaling. Moreover, inhibition of DGAT1 and DGAT2 enzymes result in a reversal of this process leading to the reinstatement of FOXO3 tumor suppressor and transcription factor function, indicating DGAT as bonified targets for resolving the negative effects of inflammatory TNF signaling on FOXO3 function.
COLON CANCER IS CURRENTLY THE 2ND MOST COMMON CAUSE OF CANCER RELATED DEATHS IN THE UNITED STATES BUT IT IS THE NUMBER ONE CAUSE OF CANCER FOR INDIVIDUALS WHO ARE NON-SMOKERS IN THE UNITED STATES. GLOBALLY, THE INCIDENCE RATES FOR COLON CANCER IN 2018 WERE NOTED AS BEING HIGHEST IN EUROPE, AUSTRALIA/NEW ZEALAND, NORTH AMERICA, AND EASTERN ASIA. FURTHERMORE, THE RATES OF COLON CANCER INCIDENCE HAVE INCREASED EQUALLY AMONG BOTH GENDERS IN DEVELOPING COUNTRIES SUCH AS URUGUAY AND SUB-SAHARAN AFRICA. ALTHOUGH INCREASED SCREENINGS HAVE BEEN IMPLEMENTED AMONG EUROPEAN UNION COUNTRIES, IN AN ATTEMPT TO REDUCE COLORECTAL CANCER CASES, IT WAS STILL FOUND TO ACCOUNT FOR THE SECOND HIGHEST NUMBER OF DEATHS AMONG EU COUNTRIES IN 2018. RECENTLY, THERE HAVE BEEN CHANGING TRENDS THAT DISPLAY A YOUNGER AGE DEMOGRAPHIC BEING DIAGNOSED WITH SPORADIC COLON CANCER IN THE USA. HOWEVER, IT IS IMPORTANT TO REALIZE THAT THIS TREND DOES NOT APPEAR TO BE A GLOBAL OCCURRENCE. THE WORLD HEALTH ORGANIZATION (WHO) AND EUROSTAT DATABASES HAVE STATED THAT THEY DO NOT PREDICT AN INCREASE OF COLORECTAL CANCER CASES AMONG A YOUNGER AGE DEMOGRAPHIC IN THE EU DURING 2019. IN ADDITION TO THIS FLUCTUATION IN AGE OF ONSET, SOUTHERN STATES SUCH AS LOUISIANA CURRENTLY DEMONSTRATE THE HIGHEST RATES OF COLORECTAL CANCER CASES IN COMPARISON TO OTHER AREAS IN THE USA.

COLON CANCER CAN BE CLASSIFIED INTO THREE MAIN CATEGORIES, WHICH CONSIST OF SPORADIC CASES, HEREDITARY, AND FAMILIAL. A MAJORITY OF COLON CANCER CASES ARE SPORADIC, WHICH MEANS THAT THEY HAVE NOTHING TO DO WITH INHERITED GENETICS BUT RATHER THEY ARE HAPPENING COMPLETELY BY CHANCE. ONLY ABOUT 5-10% OF COLON CANCER CASES HAVE A HEREDITARY CAUSE, BUT GENETIC TESTING IS ONLY USEFUL FOR THESE CASES IF THE SPECIFIC GENE IS KNOWN. IN FAMILIAL CASES, A HEREDITARY CAUSE WAS RULED OUT, BUT THERE IS MORE THAN A FAIR SHARE OF COLON CANCER
that runs in the family. However, there is not a way to genetically test these individuals because the specific underlying cause of increased colon cancer in their family history is not currently known. In essence, some colon cancer cases can be clearly associated with inherited cancer syndromes while in other cases the families have an increased risk for unknown reasons.

When looking at colon cancer risk factors, we are aware of certain things that increase an individual’s risk. For instance, inflammatory diseases of the colon such as ulcerative colitis and Crohn’s disease, clearly have an increased risk of colon cancer development. Additionally, a western diet of red meat and low fiber, as well as smoking, alcohol consumption, diabetes, and obesity have all been established as known risk factors in colon cancer development. Studies have demonstrated that mice which have been on a high-fat diet also exhibit an increased predisposition to developing colon cancer. Although the complete mechanism of these processes are not fully understood, these findings illustrate an important link that exists between obesity, lipid droplet accumulation, and colorectal cancer development.

The specific reasons as to why we are witnessing altered trends in age demographic and geographical patterns throughout the USA has not been established as of yet. However, recent findings have depicted a link between loss of FOXO3 function and increased lipid droplet (LD) accumulation in both adipose tissue and non-adipose tissue. Altered lipid droplet accumulation is a characteristic hallmark found to be associated with colorectal cancer development. At first, the simplicity of lipid droplets deemed them as solely being involved in excess nutrient storage. Although this fat storing ability does ensure a basic level of survival, this characteristic primarily serves as an indicator of how lipid droplets
have a long evolutionary history of existence. In recent years, investigators have realized that lipid droplets are involved in lipid storage processes, mobilization processes, regulatory processes, and ultimately play a strong role in the development of diseases and cancer. In combination with previous findings from our lab that have shown colon cancer proliferation to be extensively reliant upon an increased need for energy, increased LD density serves as an efficient source of energy for fueling such tumor metabolic and proliferative needs. Although lipid synthesis and storing of neutral lipids have been reviewed in various studies, the details of how this process is organized and regulated remains to be determined.

In the context of cancer, FOXO3 regulates processes such as DNA damage repair, apoptosis, cell cycle arrest via p27kip1, as well as protection from oxidative stress. Studies that compared FOXO3 levels from primary colon cancer tumors and corresponding liver metastases found that FOXO3 levels were drastically lower in the metastatic liver tumors. This finding reinforces the notion of tumorigenesis and metastasis requiring lowered FOXO3 levels. Additionally, it has been suggested that FOXO3 might participate as a co-regulator of genes involved in colorectal cancer metastasis and poor prognosis. Recent studies of FOXO3, which is a transcription factor and tumor suppressor, have illustrated that FOXO3 is inactivated in colon cancer patients. However, the link between loss of FOXO3 function and increased LDs remains to be fully understood. One possible connection could be Acyltransferase DGATs enzymes that are involved in the final stage of triglyceride synthesis (lipogenesis), yet the specific mechanisms of this regulation are unknown.
In human colon cancer cells, increased levels of Acyltransferase DGATs enzymes have been noted within previous studies conducted in our lab. Both of these enzymes are essential during the final stage of triglyceride synthesis and lipid droplet formation in the endoplasmic reticulum. Additionally, increased levels of both DGAT1 and DGAT2 were seen in colon cancer patients when FOXO3 activity was lost. Similarly, DGAT2 is a lipogenic enzyme that is found in additional cellular locations such as mitochondria-associated membranes (MAMs) and is able to translocate from MAMs to the surface of LDs themselves. Moreover, the chromosomal region that DGAT2 is located on, namely 11q13, has been linked to obesity.

DGAT1 deficient mice have been shown to demonstrate resistance to HFD-mediated obesity. Moreover, triglyceride levels in DGAT1 deficient mice were substantially lower in non-adipose tissues. These findings strongly suggest that DGAT1 levels have a proportional relationship to fat storing capacity both in adipose tissues and non-adipose tissues, regardless of diet. This further supports our notions that altering the levels of DGATs enzymes, and more specifically of DGAT1, could be implemented as a possible therapeutic target in obesity-related colon cancer.

Induction of lipid droplet (LD) formation is accomplished with the use of oleic acid (OA). To further determine the effects of lipid droplet density on FOXO3 activity, I used human colon cancer cells HT29 that were co-treated with OA/DGAT inhibitors to compare their effects with that of OA treated cells alone. As was to be expected, increased lipid droplet accumulation indeed resulted in FOXO3 translocation from the nucleus to the cytosol, ultimately leading to its degradation. I found that DGAT1/2 inhibition protected against loss of FOXO3 after oleic acid-mediated increases in LD density, indicating a
critical role for DGATs in mediating the FOXO3-LD lipogenic network. Furthermore, I found that inhibition of DGAT1/2 prevented upstream PI3K-Akt induced phosphorylation and subsequent loss of FOXO3, suggesting a regulatory loop between DGATs and the PI3K-FOXO3 axis. Functionally, I observed that inhibition of DGATs rescued expression of the FOXO3 downstream transcriptional target, p27kip1, which is typically lost after treatment of oleic acid. This exemplifies the necessity to further advance our understanding of the interplay between LDs and FOXO3 dysregulation on the cell cycle.

In patients with inflammatory bowel disease, TNFα has been found to play an essential role in maintaining intestinal inflammation which correlates to an increased risk of developing colon cancer. Additional studies built upon the idea that FOXO3 somehow plays a role in the intestinal inflammation process that predisposes an increased risk of developing colon cancer. In unison with TNFα being a major contributor to intestinal inflammation, it was found that TNFα inactivates FOXO3 through the IKK and/or PI3K pathways by causing increased phosphorylation of nuclear FOXO3. After inducing HT29 colon cancer cells with TNF for 1 and 2hr, my findings have shown there was a visible increased level of phosphorylated FOXO3 correlating with an overall loss of FOXO3. Additionally, I observed FOXO3 function was restored in cells that had been co-treated with TNF/DGAT inhibitors compared to TNF alone.

Thus, my findings demonstrate that inflammatory TNF treatment of colon cancer cells leads to reduced FOXO3 function, in part, through transactivation of PI3K-Akt signaling. Moreover, inhibition of DGAT1 and DGAT2 enzymes result in a reversal of this process leading to the reinstatement of FOXO3 tumor suppressor and transcription factor
function, indicating DGAT enzymes as bonified targets for resolving the negative effects of inflammatory TNF signaling on FOXO3 function.

Taken together, these results and data indicate that there is a critical role for DGATs enzymes in mediating the lipogenic effects of FOXO3-LDs. Although the details of these processes are not fully understood, the pattern of increased lipid droplet accumulation among colon cancer patients and the regulatory role that DGAT1/2 demonstrate within the FOXO3-LD network could be a significant element in the development of novel therapies. In both human colon cancer cells and mouse models, increased DGATs levels were found to be present. Moreover, these findings give rise to the notion of therapeutically targeting DGATs and thereby restoring FOXO3 tumor suppressor function. Further understanding of the mechanism that governs DGATs in relation to the FOXO3-LD regulatory network in colon cancer is necessary, to establish their potential role as a therapeutic target and/or potential biomarker. Furthermore, Drug resistance in colon cancer cells is an associated consequence of FOXO3 inactivation. Low levels of FOXO3 in colon cancer have recently been shown to result in chemotherapeutic resistance to cisplatin and cetuximab. Thus, reinstatement of FOXO3 function by use of DGAT1/2 inhibitors could potentially aid in chemoresistance.
**FIGURES**

**Figure 1.** In human colon cancer HT29 cells inhibition of DGATs enzyme function (DGAT1 Inhibitor A922500; DGAT2 inhibitor PF06424439) prevents OA-induced loss of FOXO3 shown by western blot (degradation) (n=3, p<0.05 *compared to Con, #compared to OA 1hr treatment).
Figure 2. In human colon cancer HT29 cells inhibition of DGATs enzyme function (DGAT1 Inhibitor A922500; DGAT2 inhibitor PF06424439) prevents OA-induced loss of FOXO3 shown by IFS (translocation from nucleus (active) to cytosol (inactive) (scale 5μm).
Figure 3. Inhibition of DGATs enzyme function attenuates OA-stimulated PI3K activation, e.g. phosphorylation of PI3K target Akt (n=3, p<0.05 *compared to Con, #compared to OA 1 hr treatment).
Figure 4. Protection of p27kip1 loss. In HT29 colon cancer cells pharmacological blockade of DGAT1/2 prevented OA stimulated loss of p27kip1, a mediator of cell cycle arrest. (n=3, p<0.05 *compared to Con, #compared to OA 1 hr treatment)
Figure 5. In human colon cancer HT29 cells inhibition of DGATs enzyme function (DGAT1 Inhibitor A922500; DGAT2 inhibitor PF06424439) prevents TNF-induced loss of FOXO3 shown by western blot.
Figure 6. In human colon cancer HT29 cells inhibition of DGATs enzyme function (DGAT1 Inhibitor A922500; DGAT2 inhibitor PF06424439) prevents TNF-stimulated PI3K activation, e.g. phosphorylation of PI3K target Akt.
FUTURE DIRECTIONS

What is the function of DGATs on growth and what mechanism is used?

TNFα is a primary inflammatory cytokine that gained initial recognition for its principle role in causing hemorrhagic necrosis in tumors. Currently, studies are aimed at gaining a deeper understanding of the pathways and mediators that are involved in TNFα-related colon cancer development. Both an intrinsic pathway and an extrinsic pathway were found to incorporate primary inflammatory cytokines, such as TNFα. The intrinsic pathway implements genetic factors that cause cancer development, while noting that such genetic events lead to the formation of an inflammatory microenvironment that is a key underlying factor in cancer development. Similarly, the extrinsic pathway implements inflammatory conditions, such as inflammatory bowel disease, as being a vital element in the facilitation of colon cancer development.

Further investigation of DGATs inhibition after TNFα treatment would be pertinent in determining whether there is cell lineage specificity. My results for the human colon cancer HT29 cells is very promising and further advancements should be made in determining the underlying functions that are associated with these findings. Perhaps other cell lineages such as HCT116, SW480, and SW620 would be useful to compare the extent to which FOXO3 function can be restored by DGATs inhibition and whether there are possible differences at varying time intervals. Similarly, perhaps there are differences that exist between individual DGAT1 inhibition versus DGAT2 inhibition among these different cancer cell lines. The underlying mechanisms that are associated with both my findings and the findings of other labs is not fully understood. Therefore, efforts should be
made to further these findings and attempt to establish useful therapeutic targets and/or biomarkers in colon cancer.
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<tr>
<th>Abbreviation</th>
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<td>5FU</td>
<td>5-Flourouracil</td>
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<tr>
<td>ACAT</td>
<td>Acyl CoA: Cholesterol Acyltransferase (ACAT)-1 and -2</td>
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<td>ACG</td>
<td>American College of Gastroenterology</td>
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<td>AFAP</td>
<td>Attenuated Familial Adenomatous Polyposis</td>
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<td>AH</td>
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<td>AKT</td>
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<td>CIN</td>
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<td>Tumor Necrosis Factor Receptor 2</td>
</tr>
<tr>
<td>TNPO1</td>
<td>Transportin 1</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>UC</td>
<td>Ulcerative Colitis</td>
</tr>
<tr>
<td>US/USA</td>
<td>United States/United States of America</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-Type</td>
</tr>
</tbody>
</table>
LIST OF REFERENCES


BIOGRAPHY

I completed my undergraduate degree at Louisiana State University. Afterward, my studies began at Tulane University School of Medicine in the two-year Masters Degree program. I will be graduating in May 2019 with my Master of Science in Molecular and Cellular Pathobiology degree.