Investigating the Cell-Autonomous Role of Autophagy in Colon Cancer and the Reliance of Mitophagy for Growth

by

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I dedicate this thesis to my mother Brenda.

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Abstract

Colorectal cancer (CRC) is the third leading cause of cancer related deaths worldwide. Cancer cells modify normal cell functions to adapt to the surrounding tumor microenvironment. Autophagy is a basic cell function used to degrade organelles, aggregated proteins, and nutrients to recycle for cellular use. In cancer, autophagy is known to play both tumor promoting and suppressive roles. In colon cancer, autophagy can enhance or inhibit tumor growth and the function is often tumor stage and context dependent. A more in-depth understanding of how autophagy alters tumor growth is necessary to better develop treatments for patients with colon cancer.

Previous literature has shown a cross-talk between epithelial autophagy and the intestinal immune response. This dissertation uncovers a novel cell-autonomous role for autophagy in colon cancer independent of the immune system. While autophagy is classically known to provide nutrients to the cell, the cellular components that are targeted for breakdown and under what context they are targeted is not known in colon cancer. Tumor cell growth is inhibited following autophagy loss, but normal colon epithelia are not impacted by inhibition suggesting a tumor selective reliance on autophagy. Under nutrient stress tumor cells employ mitophagy, a selective form of autophagy that targets mitochondria for breakdown. Inhibition of PINK/PRKN directed mitophagy significantly reduces cell growth.

Lastly, I assessed cell type specificity of autophagy in CRC. Autophagy is known to have differential roles in epithelial sub-types. In inflammatory bowel disease, loss of autophagy specifically alters Paneth cell function. To explore the role of autophagy in colon cancer, I utilized single-cell RNA sequencing to investigate changes in autophagy in epithelial cells in colon cancer. In a sporadic tumor model with loss of *Apc, p53,* and $Kras^{G12D}$ I performed single-cell analysis on colon tissue. I found that enterocytes express genes associated with increased autophagy in comparison to goblet cells or enteroendocrine cells. This data suggests a specific increase in autophagy in tumor enterocytes. Further single-cell analysis at different stages of tumor development and focus on different cell types will begin to uncover how colon tumors are modulated by autophagy.

This dissertation uncovers the cell type specificity and cell-autonomous role for autophagy in colon cancer. Further in-depth studies are needed to assess the role of tumor stage and mutational load in the requirement of autophagy in colon cancer. I have identified that tumor cells rely on mitophagy for growth and lay the foundation for therapies targeting autophagy or mitophagy in CRC

Chapter 1[1]

Introduction

Colorectal cancer (CRC) is the third leading cause of cancer related deaths in the United States. It is estimated that in 2018 over 140,000 people were diagnosed with CRC [1]. Survival rates have improved in the last three decades due to early detection, however patients diagnosed at later stages of disease have a 5-year survival rate of 14% [2, 3]. Treatments available for these patients are limited, therefore it is important to better understand tumor development and identify targeted therapies to improve overall patient care. Sporadic CRCs are typically marked by the initial loss of the adenomatous polyposis coli (*APC*) gene. APC is a scaffold protein that leads to proteasomal degradation of β-catenin. Under active cell states, WNT ligands bind to its receptor frizzled and prevent β-catenin degradation and activate target genes. Loss of *APC* constitutively activates β-catenin and causes uncontrolled epithelial proliferation. Mutations in *APC* are typically followed by sequential mutations in tumor protein *p53* (p53), and mutations in *KRAS* leading to spontaneous tumor development and progression [4]. It is well known that chronic inflammatory diseases such as Crohn's Disease or ulcerative colitis increase risk of developing colon cancer, referred to as colitis-associated cancer (CAC) [5, 6]. CACs developed from inflammatory bowel diseases (IBD) are a rare subset. Moreover, *p53*

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mutations occur earlier in the progression of CAC and *APC* mutations are less frequent and are found in late stage tumors in comparison to sporadic CRC [7]. However, CAC provides a clear link between inflammation and tumor initiation.

Autophagy is a highly regulated process that degrades and recycles cellular components. Dysregulation of autophagy is implicated in many diseases (as reviewed in [8]). Under several different cell stressors, autophagy is activated through kinase signaling and transcriptional activation by serine/threonine protein kinase 1 (ULK1) and transcription factor EB (TFEB). This activates a cascade of autophagy-related genes (ATG) [9], and formation of a spherical double layer membrane termed the autophagosome. The autophagosome delivers key cytoplasmic cargo such as organelles, foreign bodies, and cellular components to the lysosome for degradation into macromolecules that can be utilized by the cell. In CRC, autophagy is known to play tumor promoting and tumor suppressive roles [10, 11], but the underlying mechanisms are not well understood. Studies have found conflicting functions of autophagy in tumors. These discrepancies are typically due to differences in the cells and tumor models that are utilized [12-15]. Further study of autophagy and its prevalence in CRC will uncover its potential therapeutic use [16]. Here we highlight cellular pathways that regulate autophagy, selective forms of autophagy, and how these mechanisms target different cargo for degradation.

2. Autophagy subtypes

Autophagy can be classified into three major subtypes; macro-autophagy, micro-autophagy, and chaperone-mediated autophagy. There is a need for better

understanding of cellular cues and cell-dependent context by which autophagic subtypes are co-opted in cancer cells for growth and survival.

Chaperone-mediated autophagy

Chaperone-mediated autophagy (CMA) differs from macro-autophagy in that select proteins are targeted for degradation by direct targeting to the lysosome. Proteins are recognized by heat shock cognate protein 70 (HSC70). HSC70 interacts with lysosome-associated membrane protein type 2A (LAMP-2A) to internalize proteins into the lysosome. CMA substrates contain a specific motif, KFERQ, which is essential for HSC70 binding [17]. Relevant to cancer, inhibition of macro-autophagy enhances CMA-dependent degradation of mutant p53 [18]. Increased expression of LAMP-2A demonstrated activated CMA in CRC [19]. These functions highlight a potential role of CMA in tumor development. However, these processes have not been well studied in CRC.

Micro-autophagy

Micro-autophagy is the direct engulfment of cellular components by invagination of the late endosome (**Figure 1.1**). Broadly, the implications of micro-autophagy have not been studied in many cancer types. There is evidence in lung cancer that amino acid starvation, an important factor in cancer growth (discussed below), induces micro-autophagy [20]. However, the role of micro-autophagy in CRC has not been investigated in detail.

Macro-autophagy

Macro-autophagy can be broken down in to two subcategories that target cellular components for degradation; non-selective and selective. Non-selective macro-autophagy engulfs bulk cytosolic components and selective autophagy targets specific cargo for degradation (e.g. organelles and protein aggregates). For macro-autophagy, the phagophore, a precursor to the autophagosome, forms. Several ATG protein complexes are involved in early and late autophagosome formation [21]. . As the membrane is forming, microtubule associated protein 1 light chain 3 beta (LC3-I) is conjugated with phosphatidylethanolamine and is processed into LC3-II [22]. Following fusion of the autophagosome with the lysosome, which contains the required enzymes for cargo degradation, LC3-II is broken down by the lysosome [23]. The turnover of LC3-II is often used as a readout of autophagic activity [24]. Here we have provided a highly simplified overview of the complex process of macro-autophagy. This extremely coordinated event of cytoplasmic engulfment is generally activated in states of cell stress such as starvation. Anding and Baehrecke review the important role of selective-autophagic processes in maintaining cellular homeostasis in response to stress [25]. Below we highlight the known selective-autophagic pathways and their potential role in CRC (**Figure 1.1**).

Mitophagy

Mitophagy is a process that selectively degrades mitochondria [26-29]. Mitophagy is highly evolutionarily conserved [30] and is known to be activated in yeast under starvation [31]. Therefore, highly proliferative cells under nutrient stress and starvation, such as

cancer cells, may activate mitophagy. This process can be modulated by different pathways as discussed below.

Parkin-mediated mitophagy

Under normal cell homeostasis, PTEN induced kinase 1 (PINK1) is maintained on the inner mitochondrial membrane. Upon damage or stress, PINK1 moves to the outer membrane and phosphorylates parkin (PRKN). This phosphorylation allows for ubiquitination of PRKN1 and targeting to the autophagosome. Poly-ubiquitination is recognized by adapters that direct mitochondria to the autophagosome. Known adapters include sequestosome (SQSTM1), neighbor of BRCA1 (NBR1), optineurin, nuclear domain 10 protein 52, and TAX1 binding protein; although, NBR1 is found to be non-essential for PRKN-mediated mitophagy [26, 32, 33]. Through this mechanism, a recent study found that in intestinal cancers, activation of mitophagy increased CD8+ T cells [34]. The upregulation of mitophagy causes an accumulation of iron followed by permeabilization of the lysosome. This permeabilization causes the release of proteases into the cytosol that induces presentation of MHC class I on the cell surface. This presentation elicits and an anti-tumor immune response by induction of CD8+ T-cells. In colitis, pharmacological induction of mitophagy through PRKN is found to inactivate inflammasomes in macrophages and ameliorate the impact of colitis [35-37]. While there are implications for PRKN mediated mitophagy in CRC, one study found around 33% of colon tumors harbor PARK2 (the gene that encodes for PRKN) DNA copy number loss [38]. Interestingly, some colorectal cancer cell lines contain mutated forms of PRKN and may use alternate mechanisms to activate mitophagy [38]. Research is starting to

investigate how PRKN may be used as a prognostic marker in CRC as it may correlate with invasion and overall survival [39].

Parkin-independent mitophagy

Alternatively to PRKN directed mitophagy, interaction of LC3 to FUN14 domain containing 1 (FUNDC1) protein located on the outer mitochondrial membrane can also initiate mitophagy [40]. Another PRKN-independent mechanism includes BCL2 interacting protein 3 (BNIP3), a critical receptor for mitophagy [41]. In breast cancer, BNIP3 loss promoted tumor progression and metastasis [42]. BNIP3 is induced by hypoxia signaling, a critical micro-environmental stressor in CRC (discussed below)[43]. The impact of BNIP3 and mitophagy in CRC have not been studied in detail. The known function in breast cancer, and BNIP3's relationship with hypoxia signaling provides a foundation to investigate the role in CRC. Mitochondria may also be recruited to the autophagosome by FKBP8, a member of the FK506-binding protein family. FKBP8 is located on the outer membrane and has an anti-apoptotic role by interacting with Bcl-2 [44]. PRKN-independent mitophagy can be initiated by binding of LC3A to FKBP8 [45]. Additional mechanisms of PRKN-independent mitochondrial control are reviewed by Stockum et al. [46]. PRKN-independent mechanisms, and the evidence of mutated PRKN discussed above in CRC highlight the potential importance of investigating PRKN-independent mitophagy in CRC.

Pharmacological targeting of mitochondria with Mito-CP or Mito-Met_{10} in *KRAS* mutant colorectal cancers induced mitophagy and decreased cell proliferation [47]. In colon cancer, treatment with a BH3 mimetic, which inhibits Bcl-2 anti-apoptotic proteins,

induced mitophagy. Treatment with the mimetic, in combination with an mitophagy inhibitor, reduced CRC cell growth [48]. However, conflicting roles of mitophagy have been noted in cancer (Reviewed in [49]). Treatment with the mitophagy inhibitor liensinine increased breast cancer cell death [50]. Conversely, activation of mitophagy with ceramide, a molecule involved in sphingolipid metabolism, reduced tumor burden in acute myeloid leukemia [51]. In-depth understanding of mitophagy in CRC is needed in order to develop better therapies that can target mitophagy to reduce tumor growth.

Ribophagy

Ribophagy is the breakdown of ribosomes in cells, which constitute 10% of total cellular protein. Ribophagy is extremely low basally in cells [52]. Initiation of ribophagy occurs by the binding of nuclear FMR1 interacting protein 1 (NUFIP1) to ribosomes. This interaction leads to autophagosome recruitment by LC3 [53]. Starvation or molecular target of rapamycin complex 1 (mTORC1) inhibition induced NUFIP1 activity and increased ribophagy [53]. Breakdown of ribosomes under starvation underscores the importance of ribophagy for cellular nutrient maintenance. However, non-selective bulk degradation of ribosomes may also be utilized [52]. Little information is known about ribophagy in cancer. However, ribosomes contain a large amount of amino acids and nucleotides and can potentially serve as a nutrient store in the tumor environment.

Proteophagy

Clearance of proteasomes through autophagy is known as proteophagy. Cross-talk between the proteasome and autophagy is found under nitrogen starvation in cells wherein autophagy degrades ribosomes (and proteasomes) under nutrient starvation

[54]. One of the earliest citations of proteophagy suggests that this process occurs through a chaperone-mediated mechanism where the proteasome is targeted to the lysosome by HSC73 [55]. More recent work uncovers the sequestration of the proteasome in autophagosomes under cell stress, suggesting proteophagy can occur in a macro-autophagy or CMA driven fashion [56]. Currently there is no data to suggest that proteophagy is activated in cancer.

Pexophagy

Peroxisomes are small organelle structures that break down fatty acids in the cytoplasm. The degradation of these products through autophagy requires SQSTM1 and NBR1 [57]. In healthy liver, loss of autophagy through ATG7 led to a buildup of peroxisomes [58]. Under starvation conditions, ubiquitination of peroxisomes occurred by peroxisomal biogenesis factor 2 (PEX2) in HeLa cells and mouse embryonic fibroblasts [59]. To our knowledge, the utilization of pexophagy in CRC has yet to be investigated. However, hypoxia inducible factor-2α (HIF-2α), an important transcription factor in CRC (discussed below), was found to promote pexophagy in hepatocytes [60]. While these findings were not investigated in colon tissue, the activation of pexophagy under starvation and hypoxia highlights the potential importance of studying pexophagy in CRC.

Ferritinophagy

Iron storage protein ferritin is broken down by the lysosome for iron release and cellular iron utilization. This degradation is directed by the nuclear receptor coactivator 4 (NCOA4) [61]. Interestingly, ferritinophagy is required for induction of ferroptosis, a form of cell death that requires iron [62, 63]. Certain cancers have shown a sensitivity to

ferroptosis [64] and pharmacological induction of ferroptosis is found to reduce pancreatic and hepatic cancer cell growth [63, 65]. While little work has been done to investigate the importance of ferritinophagy in CRC, the essential role of iron in CRC growth [66] and the sensitivity of different cancer types to ferroptosis [67] highlights the importance in studying ferritin turnover in CRC.

Xenophagy

Xenophagy is a process initiated by the cell for protection against pathogens. Phagophores engulf pathogens and fuse to autophagosome for breakdown by autophagy. Xenophagy can play a particularly important role in the colon due to the host-microbiome interaction. Protection from intestinal epithelial infection requires the autophagy gene *ATG16L1* [68]. Recent screening of xenophagy effectors identified a V-ATPase and ATG16L1 mechanism to specifically activate xenophagy under bacterial infiltration [69]. Certain bacteria can be targeted by SQSTM1, an important protein in autophagy [70]. In Crohn's Disease, the stimulation of xenophagy using resveratrol reduced *Salmonella Typhimurium,* an enteric pathogen associated with Crohn's Disease [71]. When colon cancer cells are treated with two mircoRNAs, MIR106B and MIR93, reduced ATG16L1 prevented removal of intracellular bacteria from epithelial cells via autophagy [72]. As mentioned, patients with Crohn's Disease have an increased risk of developing CAC and this is partially due to bacterial infiltration. Understanding the role of xenophagy in host-microbiome homeostasis may be essential in characterizing the microbiota-tumor interaction.

3. Role of Autophagy in CRC

It is important to understand the role of autophagy at different stages and under different mutational loads to properly target tumors. A clinical study observed down-regulation of *ATG5* in CRC patients. However, increased expression correlated with increased incidence of invasion [73]. Conversely, expression of LC3B and SQSTM1 correlated with poor prognosis [74]. In mouse models, loss of *Atg7* in intestinal epithelial cells inhibited tumor growth through an immune response elicited by the microbiome [75]. Additionally, receptor for activated C kinase 1 (RACK1), a commonly found mutation in cancer, induced autophagy and promoted proliferation while inhibiting apoptosis in colon cancer [76]. Autophagy also modulated the degradation of the transcription factor FOXO3a in CRC. Inhibition of autophagy elevated levels of FOXO3a and led to transcriptional upregulation of pro-apoptotic genes [77]. Apoptosis also increased when autophagy was inhibited in CRC cells following activation of p53 and endoplasmic reticulum stress [78]. Conversely, treatment with Brevlin A increased autophagy and decreased tumor size [79]. This brief overview emphasizes the complexity of autophagy in CRC. It remains unclear if autophagy is anti- or pro- tumorigenic and in-depth mechanistic studies are needed. **Table 1.1** outlines some of the opposing roles of autophagy in CRC.

An understanding of mutations in autophagy associated genes, how autophagy is altered by increased mutational load, or via specific tumor suppressors or oncogenes is essential in assessing its role in tumor development. In CRC, there is low frequency of mutations in autophagy associated genes [80]. In a small cohort of patient samples, tumors expressed decreased levels of *ATG5*, however increased expression correlated

with invasion into lymphovascular tissue [73]. A study demonstrated that 95% of colon tumors expressed higher Beclin-1 compared to normal tissue [81]. These studies suggest that autophagy is important in cancer development. Similarly, in mutant *KRAS* cancers, autophagy induction occurred under starvation. Inhibition of *KRAS* reduced autophagy in these cells and inhibited cell growth [82]. In CRC-derived cell lines, p53 promoted the degradation of LC3 allowing for stable autophagic flux [83]. With loss of p53, LC3 accumulated and led to apoptosis. In CRCs with high microsatellite instability, 27% of the cancers harbored at least one mutation in either *ATG2B*, *ATG5*, *ATG9B*, or *ATG12* [84]. Additionally, a study aimed to understand responsiveness to therapy in BRAF (a protein involved in RAS/MAPK signaling) mutant colon cancers found that treatment with EGFR antibodies and checkpoint inhibitors induced autophagy and combining these treatments with an autophagy inhibitor reduced CRC cell growth [85]. Patients with Crohn's Disease have an increased chance of developing CAC. Deficiencies in the response to bacterial sensing and invasion were observed following loss of autophagy through impairment in nucleotide-binding oligomerization domain (NOD1/2) signaling [86]. Loss of autophagy or mutations in autophagic genes may increase bacterial infiltration, which can impact the development of CAC. Mutations in autophagy associated genes, or regulation of autophagy through mutations in genes such as *KRAS* and *p53*, demonstrate the important role of this mechanism in CRC. To begin addressing how autophagy can be used clinically, researchers have found a gene signature based on nine autophagy related genes that can accurately predict survival in colon cancer [87].

Histone deacetylase inhibitors as stand-alone or adjuvant therapies are currently used in several cancers [88]. In colorectal cancer cells, inhibition of autophagy through

chloroquine, in combination with the histone deacetylase inhibitor vorinostat, led to an accumulation of ubiquitinated proteins and increased cell death [89]. Additionally, chemo-resistance required decreased autophagy in 5-fluorouracil (a common chemotherapeutic for CRC) resistant cells [90]. The authors speculated, that this observation was due to low autophagy resulting in accumulation of tumor promoting oxidative stress, inflammation, and damaged mitochondria.

CRC consist of multiple epithelial cell types as well as infiltrating immune cells. In IBD it is clear that dysregulation of autophagy in Paneth cells impacts tissue injury and inflammation [91]. However, cell type specificity of autophagy in tumor growth is unclear.

Immune cell autophagy: The anti-tumor immune response can directly kill cancer cells. As the tumor progresses, the microenvironment shifts to a highly immunosuppressive state and many of the immune cells potentiate tumor growth. Immunosuppression is essential in enhancing tumor progression, and immune cells can employ autophagy to perform standard functions including antigen presentation and cytokine production (Reviewed in [92]). In tumor-associated macrophages (TAM)s, upregulation of autophagy reduced tumor growth and increased apoptosis in CRC cells. Moreover, radiosensitization of CRC required increased autophagy in TAMs [93]. Conversely, when autophagy is lost in regulatory T-cells by disrupting *Atg7,* there was impaired ability of the antitumor immune response to CRC cells [94]. This was due in part by increased apoptosis in the T-cells. While not specifically studied in colon cancer, different immune cells including neutrophils, macrophages, B-cells, and natural killer cells rely on autophagy for their development and function (Reviewed in [95]). Immune cell

specific autophagy underscore the importance of investigating this pathway in different cell types to better develop strategies for modulating tumor growth.

Epithelial autophagy: Tumor epithelial autophagy in *KRAS* driven cancers alters inflammatory mediators to suppress the immune response [96]. Furthermore, inhibition of autophagy in cancer cells blocked interferon gamma-mediated cell death [97]. The role of the immune system and its interaction with the gut microbiota is important in tumor development. Cell autonomous autophagy in healthy epithelial cells altered barrier function by breaking down junctional proteins such as claudin 2 [98]. Impaired barrier function can lead to increased bacterial infiltration to cause inflammation and damage in the gut. Recent work demonstrated tumor stage specific changes in bacterial infiltration, inflammatory signaling and cancer progression and growth in CRC [99]. This suggests a possible role of epithelial xenophagy in CRC. In CRC tumors, regulatory T-cell infiltration inversely correlated with SQSTM1 expression [100]. The utilization of autophagy in epithelial cells may alter recruitment or function of the immune response. In summary there are major differences in the direct impact of autophagy in epithelial cells, immune cells, or the heterocellular cross-talk between these cells that can impact CRC growth and progression (**Figure 1.2**). Understanding the changes in autophagy and how it impacts tumor response will allow researchers to further understand these mechanisms in different cell types.

4. Cellular cues for autophagic activation in cancer

Starvation

The highly proliferative nature of tumors leads to a reduction in availability of nutrients in the microenvironment. In cancer, hyper-activation of mTORC1, a known pathway of

nutrient sensing, contributes to cell proliferation and tumor progression. mTORC1 is activated in about 50% of CRC tumors. **Figure 1.3A** outlines the known mechanistic cross-talk in CRC between autophagy and mTORC1. In conditions where amino acids are abundant, mTORC1 is localized to the lysosomal membrane (Reviewed in [101]). Hypoxic induction of DNA damage inducible transcript 4 (REDD1) signaling has been shown to regulate mTORC1 through truncation of the hemartin (Tsc1/Tsc2) complex [102]. Since mTORC1 is activated by available nutrients, a feedback loop exists between these two mechanisms wherein autophagy generates new macromolecules to activate mTORC1. The cross-talk between these two mechanisms are essential in maintaining cell growth and proliferation [102]. Importantly, mTORC1 is integrated to the autophagic pathway via activation of TFEB and ULK1-ATG13-FIP200 (Family kinase-interacting protein of 200kDa) complex [103, 104]. Independent of mTORC1, AMPK activated the ULK1 complex under starvation [105]. Under nutrient rich conditions mTORC1 phosphorylated ULK1 and inhibited the ULK1-AMPK interaction to block autophagy [105]. It is important to consider that the TFEB, ULK1, and AMPK pathways are known to be regulated by amino acids, which contributes to another mode of autophagy regulation [106]. In a model of lung cancer, amino acid starvation led to an induction of non-selective macro-autophagy. However, amino acid starvation has been shown independent of mTORC1 to induce micro-autophagy that directly engulfs receptors of selective autophagy including NCOA4, LC3B, and SQSTM1 into endosomes [20]. The authors suggest these functions may prevent selective macro-autophagy and promote non-selective autophagy under starvation. Interestingly, under leucine starvation, a cleaved form of SQSTM1 is generated by the protease caspase-8. Under starvation when

autophagy is active, a portion of the available SQSTM1 is cleaved. In nutrient replete conditions, this cleaved protein activates mTORC1 to increase leucine sensing [107]. The cleaved SQSTM1 is not able to participate in autophagy preventing opposing functions between mTORC1 and autophagy. Moreover, in ovarian cancer cells, arginine deprivation activated autophagy to promote cell survival [108]. Inhibition of autophagy both chemically or genetically significantly reduced cell growth. While these studies were not in CRC, these findings highlight the potential of combinatorial therapeutics with autophagy inhibitors and treatments such as arginase for tumors that rely on arginine for growth [108]. Glucose uptake plays a critical role in the growth of many cancer types, including CRC. In glucose-free conditions, knock-down of autophagy associated genes increased cell death [109]. Similarly, when colon cancer spheroids were stressed under restricted glucose or serum an increase in autophagy was observed [110]. Under similar starvation conditions, *Kras* mutant tumors require autophagy for oxidative metabolism [111]. Starvation also affected expression of claudin 1 in colon cancer. Expression of claudin 1 was higher in tumor tissue and showed co-staining with lysosomal markers LAMP1 and 2 with increased autophagy. Under starvation, claudin 1 expression increased mediating a reduction in SQSTM1. This supression suggests claudin 1 cross-talks with autophagy under starvation [112]. When and how nutrient availability impacts autophagy is essential in understanding its function in CRC tumors (**Figure 1.3B**).

Hypoxia

Hypoxia plays a key role in CRC development and progression. Hypoxia signaling is mediated by two conserved transcription factors hypoxia-inducible factor (HIF)-1α and HIF-2α, which have overlapping and distinct functions. In CRC, HIF-2α

(not HIF-1α) is essential for CRC growth and progression [113]. Hypoxia is a well conserved cell stress that activates autophagy [43]. In tumor hypoxic foci, autophagy levels are highly elevated but rapidly subside upon establishment of a blood supply [114]. In colon cancer there is a known connection between hypoxia and mitophagy. Hypoxia disrupted mitochondrial respiration leading to increased mitophagy (**Figure 1.2**) [115]. Moreover, HIF-1α upregulated BNIP3 to induce mitophagy [43]. In patient derived CRC cells, inhibition of autophagy with 3-Methyladenin in combination with hypoxia, increased apoptotic death in cancer cells [116]. Moreover, the micro RNA miR-20a was found to inhibit hypoxia induced autophagy [117]. Additionally, in glioblastoma, HIF-1α induced autophagy and drove tumor growth [118]. HIF-1α does not alter CRC tumorigenesis in mouse models [113], however it will be interesting to assess if HIF-2α has overlapping roles in the context of autophagy.

Microbiota

As highlighted briefly above, autophagy can play an important role through xenophagy in managing the host-microbiome interaction. Moreover, dysregulation of autophagy is well characterized in IBD. New work studying chronic colitis suggests that autophagy protected cells by reducing apoptosis through upregulation of tumor necrosis factor-α [119]. As discussed above, the importance of autophagy specifically in Paneth cells is known [91]. In healthy tissue, induction of autophagy in Paneth cells induced interferon gamma to protect against microbiota. However, when this mechanism is lost, intestinal inflammation is exacerbated [120]. Consistent with data from IBD, the heterocellular cross-talk with microbiota is a major factor in tumor-elicited inflammation in CRC. When microbiome composition is altered under chronic inflammation or barrier

defects, changes in the inflammatory response altered tumorigenesis [121, 122]. The cross-talk between the microbiota and immune system highlights the complexity of the tumor microenvironment in the colon [123, 124]. The importance of these mechanisms have been studied in depth [125, 126]. Loss of autophagy in healthy colon epithelial cells through *Atg5* disruption altered the composition of the gut microbiota and the gut immune response suggesting implications in chronic colitis [127]. Similarly, loss of *Atg7* in intestinal epithelial cells and tumor tissue led to infiltration of anti-tumor immune cells decreasing tumor burden [75]. Treatment with antibiotics attenuated this response, further supporting a novel integration of microbiota and autophagy in tumor growth. It is important to highlight that this work utilized an *Apc* model where tumor suppressor p53 was intact. In many cancers however, p53 is deleted or mutated thus these findings may only be applicable to patients with wild-type p53 [75]. In summary, the above findings highlight the cross-talk between the microbiota and autophagy. Further mechanistic studies may uncover novel therapeutic approaches targeting autophagy and microbiota.

5. Autophagic Substrates

The broad use of autophagy to meet metabolic demands is reviewed by Rabinowitz and White [128]. Autophagy in normal cell physiology is critical to maintain amino acid levels [129]. While it is thought the products of autophagic degradation are recycled for use in cancer, in CRC the substrates targeted for autophagy and how the degradative products are utilized is not clear. In cancer, autophagy can degrade macromolecules for nutrients, and degrade tumor suppressors or oncogenes to alter growth. Below we outline both of these functions.

Previous literature has found an increase in autophagy in CRC spheroids under glucose or serum restriction [110]. However, the degradative products of this process are unknown. A study investigated this question by studying loss of *ATG5* in *RAS* driven cancer cells. Loss of *ATG5* showed global changes in the proteome. Inhibition of autophagy, in combination with starvation, increased endoplasmic reticulum chaperones, proteins involved in DNA replication, and Rig-I like receptor signaling pathway. However, proteins that are known to be essential in stress survival were not altered with autophagy inhibition under starvation conditions [96]. This work uncovers how autophagy impacts cellular response to stresses such as starvation that are observed in the tumor microenvironment. Additionally, in *RAS* driven cancers, autophagy drove glycolysis [130]. Degradation of cellular components into amino acids is essential for cancer utilization. Thomas and colleagues demonstrated that amino acid levels in starved breast cancer cells increased with activated autophagy, whereas normal cells maintained amino acid levels under starvation. It is hypothesized that this is due to the high nutrient demand to maintain the proliferation rates of the cancer cells [131]. While this study was not in CRC this underscores the importance of understanding how autophagy is used for nutrient acquisition.

While autophagy may be employed to acquire nutrients, it has been shown to break down proteins that activate or block tumor growth. Autophagy can cause the degradation of dishevelled in colon cancer and contribute to the activation of Wnt signaling, thus promoting tumor growth [132]. Similarly in CRC, the cancerous inhibitor of protein phosphatase 2a (CIP2A) is overexpressed [133]. CIP2a is involved in Myc protein stability. Temsirolimus, an FDA approved mTORC1 inhibitor that activates autophagy led

to degradation of CIP2a and cell death in CRC [134]. In colon cancer, CyclinD1 is highly expressed and contributes to hyper-proliferation. Estrogen receptor beta was shown to activate autophagy and cause the breakdown of CyclinD1 causing cell cycle arrest and tumor death [135]. To prevent growth, treatment with 4-hydroxytamoxifen caused degradation of KRAS through autophagy in colon cancer [136]. As mentioned previously, basal autophagy breaks down FOXO3a to prevent apoptosis in CRC, to promote tumor growth [77]. While some work has been done, the process of breaking down proteins to inhibit or promote tumor growth are not well studied. A thorough understanding of autophagy in the context of CRC is important in targeting these mechanisms.

6. Conclusions and Future Perspectives

In general, non-selective autophagy is used for nutrient stress while selective autophagy is used for cell maintenance. However, in the context of tumor growth in CRC or CAC, these roles may change. Understanding the autophagic substrates that are recycled and how those substrates are utilized in tumor growth and development will identify ideal targets for treatments. While we have discussed mechanisms by which tumor cells may obtain nutrients through autophagy, these mechanisms are not clearly defined in CRC. The cross-talk between hypoxia and mitophagy underscores the importance of these mechanisms in CRC. Identifying the role of selective autophagy for tumor growth will allow the development of targeted therapeutics for CRC. The potential importance of mitophagy in cell stress and nutrient availability highlights a potential target in cancers. Moreover, if tumor cells employ selective autophagy for growth and survival, these mechanisms may be targets for vulnerability in CRC. Some of these approaches

are reviewed by Martins and Baptista [137]. Additionally, we have highlighted the cell type specific contributions of autophagy and more precise work on cell type specific dependency on autophagy will shed light on the mechanistic role of autophagy in tumor development. More directly, the pathways activated or inhibited during nutrient stress and how autophagic substrates are being utilized in cancer cells will be critical to understanding the pleiotropic role of autophagy in cancer growth and progression.

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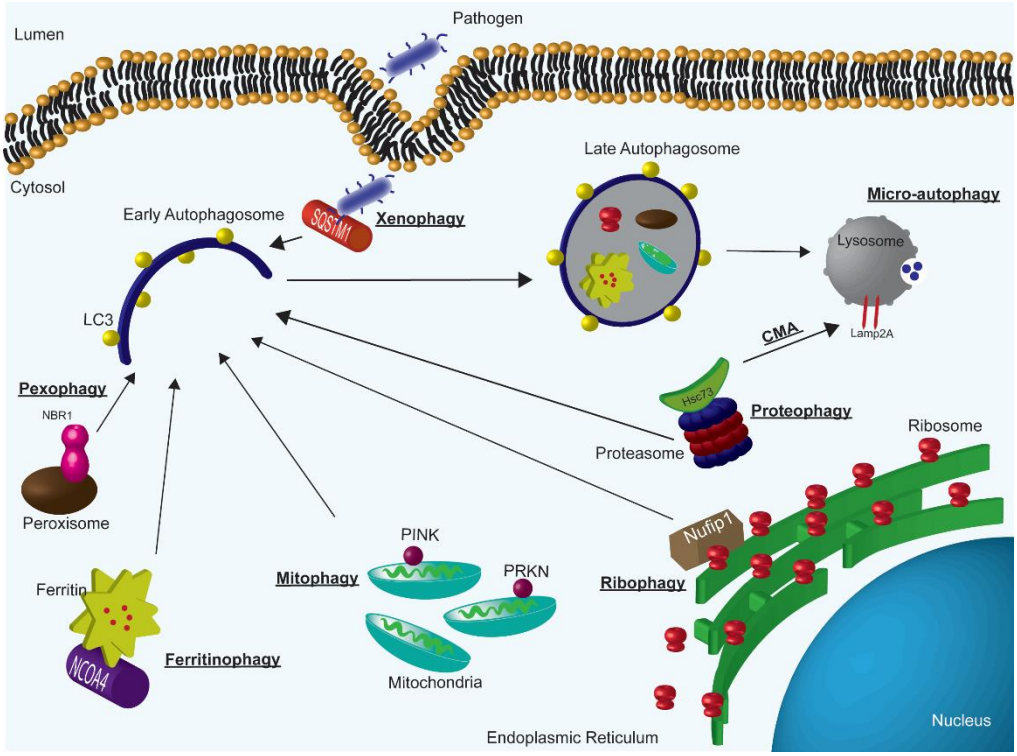

Figure 1.1. Overview of autophagy subtypes; macro-autophagy, micro-autophagy, and CMA. Specifically, highlighting examples of selective macro-autophagy.

A.

Observation	Autophagy	Tumor response	Reference
Activated chaperone mediated autophagy in tumors	Active	Pro-tumor	19
Epithelial mitophagy increases CD8+T-Cells	Active	Anti-tumor	34
Loss of PARK2 accelerates tumor development	Inactive	Pro-tumor	38
Decreased ATG5 in CRC patients	Inactive	Pro-tumor	73
Increased ATG5 yields increased invasion	Active	Pro-tumor	73
Active autophagy through LC3B and SQSTM1	Active	Pro-tumor	74
Loss of ATG7	Inactive	Anti-tumor	75
RACK1 induces autophagy	Active	Pro-tumor	76
High Beclin-1 in CRC	Active	Pro-tumor	81
Increased LC3 with loss of p53	Active	Anti-tumor	83
Autophagy suppresses immune response in KRAS cancer	Active	Pro-tumor	96
Autophagy drives glycolysis in RAS cancers	Active	Pro-tumor	130

Table 1.1A. Autophagy performs tumor promoting and tumor suppressive roles in CRC. Functions of autophagy in CRC. **A.** Observation/Autophagy indicates what mechanisms are observed in CRC tumors and if autophagy is active or inactive. Tumor response is a summary of whether or not the autophagy activity indicated generates a pro- or anti- tumor response. **B.** Summary of therapies and their modulation of autophagy. Treatment- Which therapy was employed. Autophagy- how the stated therapy modulated autophagy activation. Tumor response- how manipulation of autophagy via therapeutic treatment impacted tumor growth.

Table 1.1B.

Treatment	Autophagy	Tumor response	Reference
Mito-CP or Mito-Met10	Active	Anti-Tumor; Decreased proliferation in KRAS mutant cancers.	47
BH3 mimetic and chloroquine	Inactive	Anti-Tumor; Induced apoptosis.	48
Bafilomycin A1 or chloroquine	Inactive	Anti-Tumor; Elevated FOXO3a and transcriptional upregulation of pro-apoptotic genes.	77
Brevlin A	Active	Anti-Tumor; Promoted expression of LC3-II and induced autophagy.	79
KRAS siRNA	Inactive	Anti-Tumor; Inhibiting mutant Kras inhibits autophagy and induces apoptosis.	82
Vorinostat with chloroquine	Inactive	Anti-Tumor; Induced apoptosis.	89
5-Fluorouracil and chloroquine	Inactive	Anti-Tumor; 5-FU treatment induced autophagy for resistance. Inhibition of autophagy reduced growth.	90
Temsirolimus	Active	Anti-Tumor; Inhibited mTOR to activate autophagy and degrade CIP2A.	134
Estrogen Receptor Beta	Active	Anti-Tumor; Autophagy directed CyclinD1 degradation inhibited growth.	135
4-Hydroxytamoxifen	Active	Anti-Tumor; Degradation of KRAS through autophagy induced cel death.	136

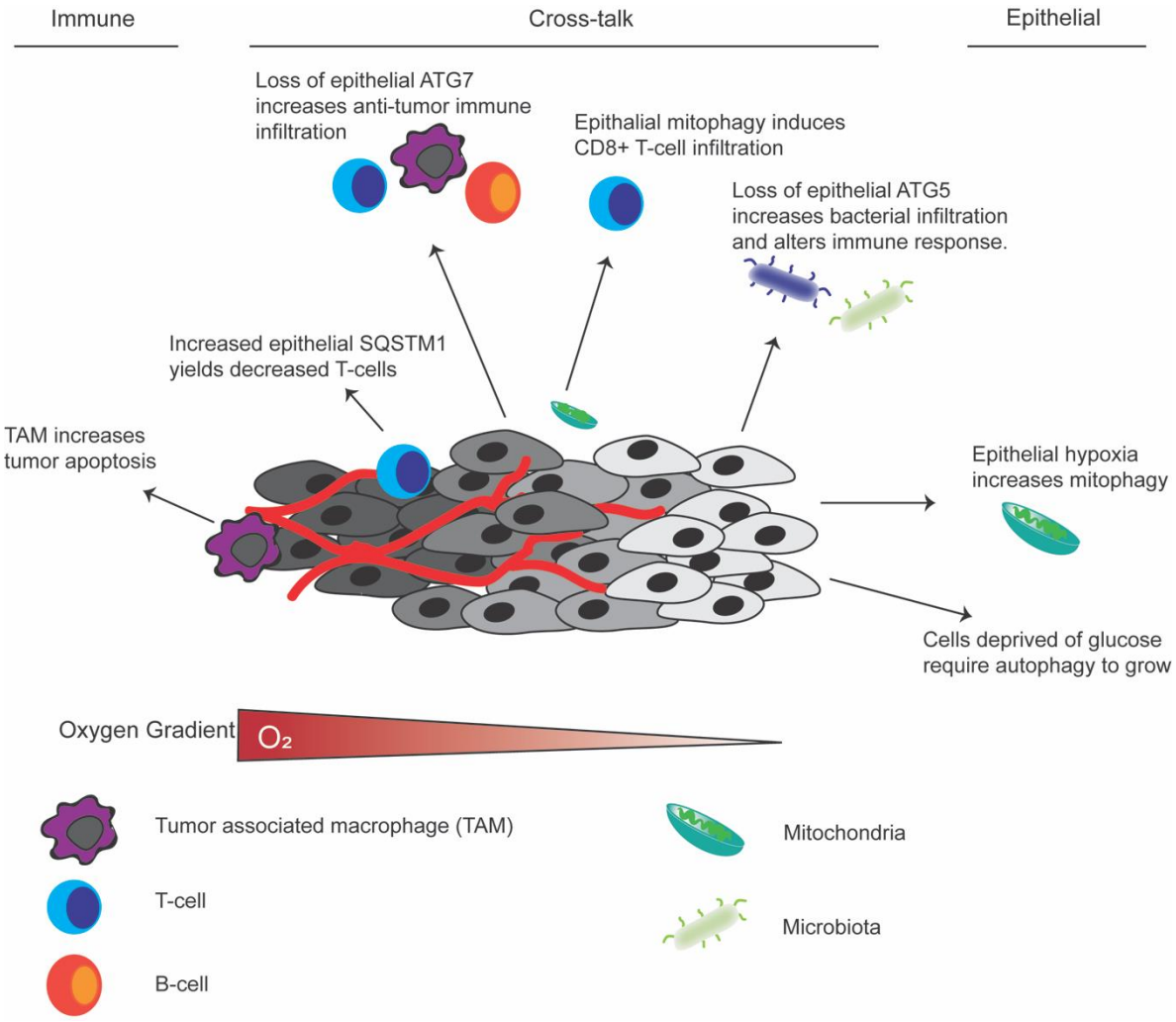

Figure 1.2. Cell-autonomous and non-autonomous cures of autophagy in CRC. Schematic of the tumor microenvironment highlighting the impact of autophagy. Cell autonomous roles of autophagy in immune, epithelial or, the cross-talk between cell types in colorectal cancer.

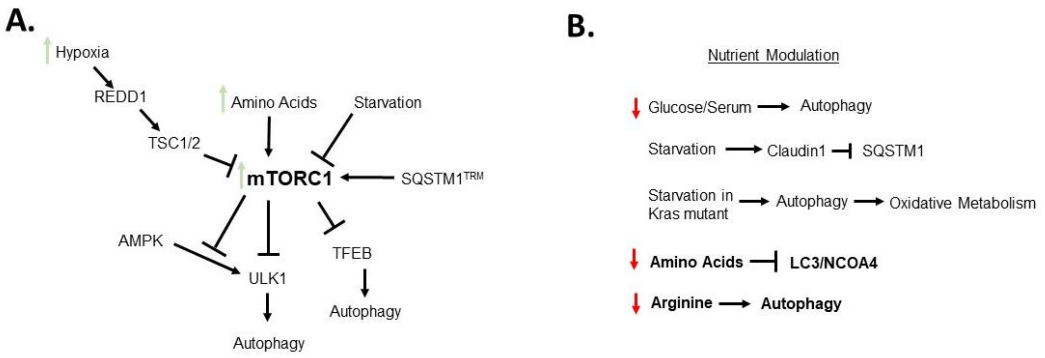

Figure 1.3. Mechanisms of mTOR and nutrient modulation impacted by autophagy. Schematic summarizing **A)** Simplified overview of mechanisms of mTORC1 regulation. and **B)** how nutrient modulation impacts autophagy. Bolded mechanisms indicate data from non-CRC samples. Please refer to text for detailed mechanisms.

Chapter [2]

Colorectal Cancer Cells Rely on Mitophagy for Tumor Growth.

Abstract

Cancer cells re-program cellular metabolism to maintain adequate nutrient pools to sustain proliferation. Moreover, autophagy is a regulated mechanism to breakdown dysfunctional cellular components and recycle cellular nutrients. However, the requirement for autophagy and the integration in cancer cell metabolism is not clear in colon cancer. Here we show a cell-autonomous dependency of autophagy for cell growth in colorectal cancer. Loss of epithelial autophagy inhibits tumor growth in both sporadic and colitis associated cancer models. Genetic and pharmacological inhibition of autophagy inhibits cell growth in colon cancer-derived cell lines and patient-derived enteroid models. Importantly, normal colon epithelium and patient-derived normal enteroid growth was not decreased following autophagy inhibition. To couple the role of autophagy to cellular metabolism, a cell culture screen in conjunction with metabolomic analysis was performed. We identified a critical role of autophagy to maintain mitochondrial metabolites for growth. Under stress, cancer cells activate mitophagy to

[2] This chapter represents a submitted manuscript: Devenport SN**,** Singhal R, Taranto JG, Kerk S, Oravecz-Wilson K, Greenson JK, Soleimanpour SA, Reddy P, Lyssiotis CA, Shah YM. Colorectal cancer cells employ mitophagy for nutrient acquisition under cell stress. *Under Review Genes and Development*

access nutrients for growth. Loss of mitochondrial recycling through inhibition of mitophagy hinders colon cancer cell growth. These findings have revealed a novel cell-autonomous role of autophagy that plays a critical role in regulating nutrient pools *in vivo* and in cell models and provides new therapeutic targets for colon cancer.

Introduction

Autophagy is an important process involved in maintaining cellular homeostasis. Autophagy removes defective organelles and proteins through lysosomal break down. This process can occur via macro-autophagy (herein autophagy); the non-selective engulfment of cytoplasmic contents, or through selective autophagy which targets specific cargo. In colon cancer, autophagy is found to have both pro- and anti- tumor functions in cancer-derived cell lines [1-3]. Consistent with this data, studies have also found both beneficial and deleterious roles of autophagy in clinical outcomes in colorectal cancer (CRC) patients [4-6]. Therefore, the function of autophagy in colorectal cancer (CRC) currently remains unclear. In mouse models of colon cancer, intestinal epithelial disruption of *Atg7*, a gene involved in formation of the autophagosome membrane, led to decreased tumors [7]. The work demonstrated that intestinal epithelial inhibition of autophagy promoted an anti-tumor immune response via alterations in the commensal microbiota population. This data is consistent with changes in the basal gut microbiota following intestinal epithelial *Atg5* disruption [8].

The tumor microenvironment increases cell stress caused by decreased oxygen availability, reduced nutrient supply, and anti-tumor immune response. To adapt to limited oxygen and nutrients, cancer cells modify metabolic pathways to maintain growth. One mechanism is through utilizing autophagic products to replenish nutrient pools in cancer [9-15]. However, all of this work has been done in KRAS mutant tumors and very little is known with respects to contribution and integration of cellular autophagy to colon cancer cell metabolism and growth.

In the current study, we identified a cell-autonomous dependency of autophagy in colon cancer cell lines, patient-derived enteroids, and mouse models. Loss of epithelial autophagy in murine tumor models reduced overall tumor number, tumor burden, and proliferation. Consistent with these data, tumor growth and proliferation were significantly decreased in CRC-patient derived enteroid models, but not in normal enteroids. In nutrient starved environments, colon cancer cells require autophagy to maintain cellular nutrient pools. Through metabolomics and lysosomal proteomics, mitophagy was rapidly initiated in low nutrient conditions and recycling of mitochondrial metabolites was observed. Temporal knockdown of mitophagy led to decreased colon cancer cell growth in nutrient rich cell culture conditions. These data demonstrate that CRCs are addicted to mitophagy to maintain cell growth. There are several clinical trials targeting autophagy for cancer treatment, and this work establishes a critical role of mitophagy in CRC growth [16].

Results

Intestinal epithelial disruption of autophagy inhibits colon tumor growth.

Atg5$^{fl/fl}$ mice were crossed to mice expressing Cre recombinase from the Villin promoter to specifically target intestinal epithelial cells. The azoxymethane (AOM) and dextran sulfate sodium (DSS) model is an established colitis associated cancer (CAC) model that specifically develops colon tumors. In the AOM/DSS model, *Atg5*$^{fl/fl}$ and *Villin*^{Cre}*;Atg5*$^{fl/fl}$ mice showed no significant difference in body weight, although a slight decrease was noted in the *Villin*^{Cre}*;Atg5*$^{fl/fl}$ mice during the final cycle of DSS (**Figure 2.1A**). The *Villin*^{Cre}*;Atg5*$^{fl/fl}$ mice demonstrated a decrease in tumor number, size, and burden (**Figure 2.1B**). Tumors from *Villin*^{Cre}*;Atg5*$^{fl/fl}$ mice had reduced proliferation as measured by Ki67 staining (**Figure 2.1C&D**). However, we did observe in the few rare large tumors from the *Villin*^{Cre}*;Atg5*$^{fl/fl}$, that proliferation was comparable to *Atg5*$^{fl/fl}$ mice (**Figure 2.1C&D**). Previous work investigating loss of *Atg7* in intestinal epithelial cells highlighted the impact of the immune response and gut microbiota in tumors [7]. Cytokines and chemokines mRNA were measured and no change was found between the *Atg5*$^{fl/fl}$ or *Villin*^{Cre}*;Atg5*$^{fl/fl}$ mice (**Figure 2.1E**). Similarly, loss of intestinal epithelial autophagy did not alter disease susceptibility to acute colitis induced by DSS. No changes in weight, colon length, or inflammation score as determined by a blinded pathologist were noted (**Figure S2.1 A-D&F**). Expression of cytokines and chemokines was not altered with loss of autophagy (**Figure S2.1E**). Transcription factor EB (TFEB) activates genes involved in autophagosome formation, cargo recognition, and fusion with the lysosome. When autophagy was disrupted by loss of TFEB in a tamoxifen inducible *Vil-ER*^{T2}*;Tfeb*$^{fl/fl}$ model, there was no change in weight, colon length, or

inflammation score (**Figure S2.1 G-J&L**). However, select inflammatory mediators including *Tnfα*, *Cxcl1*, *Cxcl2*, *Il10*, and *Cdllb* were significantly increased (**Figure S2.1K**). While we did not observe effects of autophagy loss on the response to acute colitis, others have clearly demonstrated a role for intestinal epithelial autophagy in colitis severity [17-19]. Genome wide association studies have linked polymorphisms of many known autophagic genes to susceptibility for ulcerative colitis or Crohn's Disease [20, 21]. Moreover, the data from the *Vil-ER^{T2}Cre;Tfeb$^{fl/fl}$* model showed increased pro-inflammatory mediators following injury. Therefore, the development of tumors through AOM/DSS is confounded by an inflammation driven tumor development. A sporadic colon tumor model was generated by crossing the *Apc$^{fl/fl}$* or the double *Apc$^{fl/fl}$;Atg5$^{fl/fl}$* mice to a tamoxifen-inducible colon specific *Cdx2-ER^{T2}Cre [22].* Mice were induced with a single dose (50mg/kg) of tamoxifen and 6-weeks following injections, tissues were collected. Mice showed no difference in body weight (**Figure 2.2A**). The *Cdx2-ER^{T2}Cre;Apc$^{fl/fl}$;Atg5$^{fl/fl}$* showed a significant reduction in tumor number and burden compared to *Cdx2-ER^{T2}Cre;Apc$^{fl/fl}$* mice (**Figure 2.2B**). Proliferation measured by Ki67 was reduced with loss of *Atg5* (**Figure 2.2C**). Adjacent normal tissue showed no difference in proliferation with autophagy loss (**Figure 2.2D**). To investigate if infiltration of immune cells was altered in the sporadic model following loss of autophagy, flow cytometry analysis of abundant immune populations were assessed. Two weeks following tamoxifen induction, immune cells were isolated from the colon. No difference was observed between the relative monocyte, T-cell, or neutrophil populations (**Figure 2.2E**). In inflammatory bowel disease (IBD), Paneth cells are particularly impacted by changes in autophagy [23, 24]. Therefore, we performed gene expression analysis of

Paneth cell markers in our *Cdx2-ER^{T2}Cre*;*Apc$^{fl/fl}$*;*Atg5$^{fl/fl}$* cohort and found no changes in Paneth cells with loss of autophagy in either tumor or matched normal tissue (**Figure S2.1M**).

Autophagy loss inhibits tumor proliferation in a cell-autonomous manner.

CRISPR/CAS9 mediated disruption of TFEB in CRC-derived HCT116 cells (**Figure 2.3A**) showed a marked reduction in growth as assessed by MTT and long-term clonogenic cell survival assays (**Figure 2.3B-D**). In addition, doxycycline inducible shRNAs for TFEB in HCT116 and SW480 cell lines demonstrated reduced growth (**Figure 2.3E-K**). Empty vector controls are not impacted by treatment with doxycycline (**Figure S2.2A**). ATG4B is an essential regulator of autophagy [25]. Stable HCT116 cells expressing a dominant negative ATG4B^{C74A} mutant demonstrated decreased growth by MTT and clonogenic analysis (**Figure S2.2 B&C**) [26]. Pharmacological inhibition of autophagy is currently in clinical trials for a number of cancers (NCT02333890; NCT02378532; NCT03400865). To understand the impact of pharmacological inhibition, growth in CRC-derived cell lines was measured following treatment with chloroquine, a lysosomal inhibitor. In CRC-derived cell lines (SW480, HCT116, DLD1), increasing doses of chloroquine led to marked reduction in cell growth (**Figure 2.4A**). Similar response was observed in CRC-derived HT29, RKO, and mouse MC38, and CT26 cell lines (**Figure S2.3A**). Autophagy can be activated by serine, threonine protein kinase 1 (ULK1) [27]. Inhibition of ULK1 with SBI-0206965 also reduced cell growth similar to chloroquine (**Figure S2.3B**). Cell growth was rescued when low dose (but not high dose) chloroquine was removed (**Figure S2.3C**).

To assess if the impact of autophagy loss to cell growth was selective to tumor cells, four patient-derived tumor enteroids and two normal colon enteroids were assessed [28]. Enteroids 282,584, and 590 are adenomas located in the ascending colon and enteroid 245 is an adenoma: sessile serrated from the cecum. Patient-derived tumor enteroids demonstrated significant growth inhibition following chloroquine treatment, where normal colon enteroids did not demonstrate any growth defects following inhibition of autophagy (**Figure 2.4B-C and S2.3D**). It is interesting to note that a sessile serrated tumor enteroid did not respond to autophagy inhibition. Sessile serrated tumors are a recently recognized class of colon cancers that present with BRAF mutations compared to APC mutations that are seen in the majority of colon cancer [29, 30]. The inhibition of growth highlights a dependency on autophagy in tumor cells that is not observed in normal tissue.

Tumor cells rely on autophagy under states of limited nutrient availability.

To understand if the dependency of autophagy in tumor cells is linked to cellular metabolic demands, we established a low dose of chloroquine or low nutrient conditions that did not alter cell growth (**Figure 2.5A&B and S2.4A**). Cells cultured in a low nutrient condition in combination with low dose chloroquine significantly decreased cell growth (**Figure 2.5C**) compared to either treatment alone. To understand the cellular metabolic demand that require autophagy, we heat inactivated serum at 95 C (herein $Serum^{HI}$) compared to the standard 52 C to remove heat labile nutrients. Similar to reduced serum, $Serum^{HI}$ combined with autophagy loss reduced cell growth (**Figure 2.5D**). Glucose or iron depletion did not have an additive or synergistic effect on cell

growth in combination with autophagy inhibition (**Figure S2.4B&C**). Moreover, supplementing insulin and epidermal growth factor (EGF) did not rescue the growth defect (**Figure 2.5E&F**). The additive effect of autophagy loss with $Serum^{HI}$ was similar following ULK1 inhibition (**Figure S2.5A**). To identify which metabolites were impacted under autophagy loss in combination with nutrient stress, the intracellular metabolomes of SW480 cells treated with $Serum^{HI}$ or chloroquine at 2.5µg/mL or co-treated with $Serum^{HI}$ or chloroquine for 2-days were analyzed via liquid chromatography/mass spectrometry (LC/MS) (**Figure 2.5G**). This time point was selected as no change in growth is observed at 2-days (**Figure 2.5D**)**.** Interestingly, we found only slight changes in the metabolome with either treatment alone, consistent with our growth data However, co-treatment led to significant changes in several metabolites. Metabolites which were significantly changed in the $Serum^{HI}$ and chloroquine group were analyzed for pathway analysis using Metaboanalyst [31]. A significant mitochondrial metabolite signature was found (**Figure 2.5G-H**). However, supplementation of individual metabolites did not rescue the growth defects (**Figure S2.6A**). This suggests that a combination of metabolites is important in altering cell growth.

<u>Colorectal cancer cells use mitophagy to meet cellular metabolic demands.</u>

Alterations in metabolites involved with the TCA cycle suggested an impact on mitochondria. Mitochondria can be targeted by autophagy through a process of selective autophagy known as mitophagy [32]. To assess if mitophagy is essential in CRC to meet the metabolic demands for proliferation, mitophagy flux was assessed in CRC-derived cell lines. The mitochondrial specific protein cyctochrome c oxidase

subunit 8 (COX8) fused to two fluorescent reporters, mCherry and green fluorescent protein (GFP) (COX8-mcherry-GFP) was used. If mitochondrial are targeted to the lysosomes GFP fluorescence is quenched upon a change in pH, where mCherry fluorescence remains (**Figure 2.6A**) [33]. Using flow cytometry in HCT116 and SW480 expressing Cox8-mCherry-GFP and cultured in $Serum^{HI}$ conditions demonstrated an increased flux in mitophagy following nutrient stress (**Figure 2.6B**). To further validate this observation, proteomic analysis was performed in lysosomes in control or $Serum^{HI}$ conditions. A stable TMEM192 expressing HCT116 cell line was established to enrich for lysosomes via immunoprecipitation using a LysoIP method (**Figure 2.6C&D**) [34]. Lysosomal proteomics demonstrated an enrichment of mitochondrial proteins in the cells under $Serum^{HI}$ (**Figure 2.6E**). The total lysosomal proteome content consisted of ~8% mitochondrial proteins, in which 90% of all mitochondrial proteins identified where higher in the lysosomes of $Serum^{HI}$ treated cells. This data demonstrates that mitophagy is integrated with the cellular nutrients needs and is upregulated during nutrient stress.

Mitophagy is essential for CRC growth.

To understand the contribution of mitochondrial targeting to the lysosome for CRC growth mitophagy was genetically inhibited. PTEN-induced kinase 1 (PINK1) is important for inducing mitophagy [35]. PINK1 is involved in PINK1/Parkin (PRKN) mediated mitophagy and phosphorylates PRKN which is then polyubiquitinated and targeted for autophagic degradation. We generated doxycycline inducible shRNA constructs targeting PINK1 in SW480, HCT116, and RKO cells (**Figure 2.7A-C**). Knockdown of PINK1 in these cell lines significantly reduced growth when assessed by

MTT assay (**Figure 2.7A-C and S2.7E)**. Similarly, knockdown of PRKN in HCT116 and RKO significantly reduced growth when assessed by MTT and clonogenic assay (**Figure S2.7A-E**).

Discussion

Autophagy is a cellular process that allows for the sequestration and breakdown of organelles and cellular components. Autophagy is found to be both pro-and anti-tumorigenic [36-38]. Heterocellular crosstalk exists between tumor epithelium and the microenvironment, and current work in CRC mouse models demonstrates an important role of epithelial autophagy in sustaining an immunosuppressive environment via gut commensals [7]. Importantly, the activation of autophagy in colon cancer is found to be context dependent on microbial infiltration, inflammation, and tumor stage [7, 39-42]. While autophagy is often thought to be a mechanism for nutrient recycling, or degradation of dysfunctional organelles, the precise role in colon cancer is not known. Specifically, the metabolic cues which activate autophagy, and the cellular metabolites which autophagy provide to maintain growth have not been investigated in colon cancer. We have shown that loss of autophagy through ATG5 inhibits tumor growth in a cell-autonomous fashion in inflammation-driven (AOM/DSS), sporadic (*Apc)*, and patient derived *in vitro* models of CRC. Mechanistically we show that under nutrient stress autophagy is directly integrated to meet nutrient demands via mitophagy.

In two CRC tumor models, we observed no changes in immune cell infiltration or immune signaling as previously described [7]. The lack of immune response in our models could potentially be due to the differences in the functions of ATG5 and ATG7. Autophagy associated genes, including ATG7 are found to have autophagy independent functions [43]. Other differences may be attributed to experimental design. For our experiments we use littermate controls and standardized the microbiome by mixing the bedding prior to tumor induction to prevent potential microbiota differences [44]. It is also documented that microbiota differ based on housing facilities [45]. While

other studies have identified immune differences, our experimental design and potential microbial differences allowed us to highlight the cell-autonomous role of autophagy in tumor development.

The hyper-proliferative nature of tumor cells reprograms cellular metabolism and activate pathways to replenish nutrient pools in tumor cells. In pancreatic cancer, cells scavenge for extracellular proteins to acquire amino acids [46]. Breast cancer utilizes autophagy under starvation to maintain amino acid levels [47]. In our study, we have identified autophagy as a key function that colorectal cancer cells rely on for proliferation. Our *in vitro* cell models are cultured in a highly nutrient rich medium. Upon a challenge with pharmacological or genetic autophagy inhibition, growth is dramatically reduced. This suggests that colon cancer cells are addicted to autophagy for growth and have adapted to rely on this mechanism for proliferation. To integrate autophagy to cellular metabolic demands, we found that loss of heat labile nutrients in serum, (but not iron or glucose), led to a robust decrease in cell growth in combination with autophagy inhibition. However, these results suggest that nutrients acquired through autophagy, and more specifically, mitophagy are required for general cell maintenance in tumors. This is supported by the basal levels of mitophagy that we observed in CRC cells under nutrient rich culture conditions. While we were unable to rescue growth with individual supplementation of nutrients, it is possible that the combination of nutrients acquired through mitophagy are required for cell proliferation. The metabolomics data, the rapid decrease in cell growth when autophagy and mitophagy are inhibited, and a potentiation of reduced cell growth in combination with nutrient stress suggest that a major role of mitophagy is to replenish the nutrient pool in cancer cells. However, a decrease in

growth could also be due to reduced recycling of defective mitochondria. Moreover, autophagy is essential in regulation of proteins critical for cell growth [48]. Future work is focused on decoupling the importance of nutrient recycling to other autophagic functions in colon cancer cell growth.

To clearly understand the role of autophagy in CRC, patient-derived enteroid models and adjacent normal enteroids were utilized. Patient-derived tumor enteroids [28] treated with chloroquine showed a marked decrease in growth when compared to patient derived normal enteroids. The tumor selective response further highlights the essential role of autophagy modulation in tumor growth. Interestingly, we observed no growth inhibition in the BRAF mutant (Val600Glu) enteroid model. BRAF mutations are present in about 10% of patients [49]. This particular enteroid was generated from a sessile serrated tumor [28] and BRAF mutations are known to be drivers for this tumor type [29, 30]. We are not aware of any literature that investigates the functional role of autophagy in sessile tumors but this finding uncovers the importance of understanding autophagy under different mutational burdens. Furthermore, it is important to consider the mutational load present within the models used in our study and others. p53 is mutated in about 50% of colon cancers [49]. However, the ATG7 model discussed above [7], and our AOM/DSS and sporadic tumor models, typically do not harbor p53 mutations [50, 51]. Extensive work is needed to understand the genotypic variability in CRC to autophagy inhibition.

We have identified mitophagy as an important selective pathway for nutrient acquisition in colon tumors. Mitophagy is a newly studied modulator of cancer growth and its particular role in colon cancer is not well understood. A study identified DNA

copy number loss of PRKN (*PARK2* gene) in about 33% of the colon tumors screened. *PRKN* deletion enhanced tumor growth in *Apc*$^{+/Min}$ mice. In addition to PRKN being important in mitophagy, PRKN is an E3 ubiquitin ligase for cyclin E. Loss of PRKN led to an increase in cyclin E and progression of the cell cycle [52]. Moreover, mitophagy in tumor epithelium was shown to activate $CD8^+$T-cells to reduce tumor burden in the colon [53]. The cell-autonomous role of mitophagy was not directly assessed on cell growth. Here, our work outlines a novel role for PINK/PRKN mediated mitophagy in an immune cell-independent context. Clinically, the expression of PRKN is prognostic in patient outcome. Decreased PRNK expression is correlated with increased survival [54], however increased expression is found with enhanced invasion in tumors [54]. It is possible that the role of mitophagy varies dependent on stage or spectrum of mutations in CRC. It is also important to consider that PINK1-PRKN independent mechanisms of mitophagy exist [55-57]. The use of pharmacological tools to target mitophagy are already in development for cancer treatments. In Kras mutant CRC, treatment with pharmacological inhibitors of mitochondria, Mito-CP and Mito-$Met_{10,}$ decrease cell proliferation [58].

This work underscores the importance of autophagy in nutrient acquisition in colon cancer and the potential for mitophagy inhibition to be used alone or in combination with other chemotherapeutics to improve overall colon cancer outcomes.

Methods

Mouse experiments: For all experiments, male and female mice, 6 to 8-weeks of age were used. All mice are a C57BL/6 background. ATG5 TM1a conditional ES cells were acquired from Riken and the mice were generated by the University of Michigan Transgenic core. Microbiome was normalized for 1-2 weeks prior to experiment initiation by combining bedding and distributing evenly among experimental mice. DSS experiments were completed by placing mice on 2.0% DSS in water for 7-days followed by a 3-day recovery on regular drinking water. For AOM/DSS experiments, mice were injected intraperitoneally (I.P.) with 10mg/kg of AOM. Five days after injection, mice were cycled on and off 2.0% DSS in their drinking water for one week followed by a two-week recovery as previously described [59]. Weights were taken daily. For spontaneous tumors (*Cdx*^{ERT2};*Apc*$^{fl/fl}$;*Atg5*$^{fl/fl}$), mice were injected with a single dose (50mg/kg) of tamoxifen. Six weeks later tissue was collected. Tumor burden is a summation of total tumor volume per mouse.

Histology and immunofluorescence: Histological analysis was scored by a blinded pathologist as previously described [60]. Tissues were collected and fixed in 10% formalin for 24-hours followed by embedding in paraffin. 5μM sections were stained for H&E. Immunofluorescence of Ki67 (1:100; Vector Labs), was completed using antigen retrieval in sodium citrate (Tri-sodium citrate 11.4mM, pH 6.0, 0.05% Tween-20) and labelled with (Alexa 488, ThermoFisher). Tissue was mounted with ProLong Gold with DAPI (Invitrogen). Images were quantified using ImageJ software as percent positive Ki67 area to DAPI positive.

RNA isolation and qPCR analysis: RNA was isolated using TRIzol chloroform extraction. RNA was reverse transcribed using MMLV reverse transcriptase (ThermoFisher). qPCR analysis was done using the listed primers (**Table S2.1**) and Radiant Green qPCR master mix (Alkali Scientific Inc.).

Enteroid Culture: Enteroids were cultured as previously described [28]. Lines 87 and 89 were cultured in completed LWRN medium. Additional lines (282,584,590,245) were cultured in Kerotinocyte Growth Medium (ThermoFisher). Cultures were plated in Matrigel (Corning) and allowed to establish for at least 3 days. Following establishment cells were treated either with control (Sterile PBS) or chloroquine at 75µg/mL (in PBS) for 3 days. Images were taken at 24 and 72-hours post treatment. Measurements were completed by normalizing the relative area of an individual enteroid to day 0. All measurements were completed by a blinded observer.

Flow Cytometry: Analysis was done using FlowJo software. For Cox8-mCherry-eGFP, BioRad Ze5 Cell Analyzer was used. Cells were first sorted for mCherry positivity followed by eGFP. Flow cytometry analysis of immune cells was done using the Beckman Coulter MoFlo Astrios, immune cells from the colon were isolated by 25mM EDTA digestion to remove epithelial cells followed by a 0.5mg/mL collagenase IV digestion and were enriched for using a 40% to 70% percoll gradient. Immune cells were stained for with CD45 Alexa Fluor 780, 1:200 (eBioscience) , CD4 PECy7 1:300 (Affymetrix), Cdllc FITC 1:200 (Biolegend), Cdllb APC 1:250 (eBioscience), Ly6C V450

1:300 (BD Bioscience), Ly6G PE 1:300 (BD Bioscience), F4/80 BV510 1:100 (BD Bioscience), 7AAD Percp Cy 5.5 1:300 (BD Bioscience).

MTT assays: 24-hours following plating a Day 0 reading was taken. Cells were incubated for 45 minutes with Thiazolyl Blue Tetrazolium Bromide (Sigma) then solubilized with dimethyl sulfoxide. Absorbance was read at 570nm. Following the Day 0 read, the corresponding treatment and readings were taken every 24-hours for 72-hour assay or every other day for 6-day assay. All reads were taken in technical triplicates.

Protein isolation and Western Blotting: All protein samples were separated by SDS-PAGE and transferred on to nitrocellulose membrane. Antibodies were used as follows. TFEB 1:1000 (Bethyl), LC3B 1:1000 (Cell Signaling), ATG5 1:1000 (Cell Signaling), HA-Tag 1:1000 (Abcam), LAMP1 1:1000 (Cell Signaling), Lamin AC 1:1000 (Active Motif), GAPDH 1:1000 (Santa Cruz), β-actin 1:1000 (Proteintech), PRKN 1:1000 (Cell Signaling).

Cell lines: All cell lines were cultured in DMEM with 10% fetal bovine serum unless otherwise noted. Stable *TFEB* knockout line was generated using gRNA in Lenticrispr V2 (Feng Zhang; Addgene plasmid 49535) [61] using the guides listed (**Table 2.1**). Constructs for doxycycline inducible shRNA were generated using the Tet-pLKO-puro (Dmitri Wiederschain; Addgene plasmid #21915). Plasmids were generated and inserted in to a lenti-viral vector for stable transfection. Knockdown was induced using 200ng/mL of doxycycline for 48-hours. The HCT116 cells used for tracking mitophagy

were generated from the pCLBW Cox8-mCherry-EGFP plasmid (David Chan;Addgene plasmid #78520). ATG4B mutant expressing cell line was developed by stable expression of pmStrawberry-Atg4B^{C74A} (Tamotsu Yoshimori; addgene plasmid #21076). We generated the HCT116 LysoIP line using the pLJC5-Tmem192-3xHA (David Sabatini ;Addgene plasmid # 102930). Cells were treated chloroquine diphosphate (Sigma) and SBI-0206965 (Cayman Chemical) using concentration and time as shown in the figure.

Metabolomics: Polar metabolites were extracted in ice cold 80% methanol on dry ice for 10 minutes. Proteins and cell debris were precipitated by centrifugation at 13k rpm for 10 minutes at 4C. Metabolite supernatants were dried on a SpeedVac and submitted for steady state metabolomics profiling [62, 63]. An Agilent 1290 Infinity II LC -6470 Triple Quadrupole (QqQ) tandem mass spectrometer (MS/MS) system was used. For negative ion acquisition, a Waters Acquity UPLC BEH amide column (2.1 x 100mm, 1.7µm) was used with the mobile phase (A) consisting of 97% water, 3% methanol 10 mM tributylamine, 15 mM acetic acid, and 5 µM Agilent infinity lab deactivator additive and mobile phase (B) 10mM tributylamine, 15mM glacial acetic acid, 5 µM Agilent infinity lab deactivator additive. Pump A and C deliver buffer A and B respectively. Pump D delivers acetronitrile to wash the column at the end of the run. The following gradient was used: 0-2.5 min, 100% A at 0.25 mL/min (till 27 min for the analytical run); at 7.5 min, 80% A; at 13 min 55% A; at 20 min, 1% A and kept to 24.0 min; at 24.05-27 min, 1%A and 99% D; at 27.05-31.35 min, 1%A and 99% D at 0.8 mL/min flow rate; at 32.25 to 39.9 min, 100%A at 0.40 mL/min flow rate; at 40 min 100%A, 0.25 mL/min. The column was kept at 40 ºC and 3 µL of sample was injected into the LC-MS/MS with a

flow rate of 0.2 mL/min. Tuning and calibration of the QqQ was achieved through Agilent ESI Low Concentration Tuning Mix.

The MassHunter Metabolomics Dynamic MRM Database and Method was used for target identification. Key parameters of AJS ESI were: Gas Temp: 150℃, Gas Flow 13 L/min, Nebulizer 45 psi, Sheath Gas Temp 325℃, Sheath Gas Flow 12 L/min, Capillary 2000 V, Nozzle 500 V. Detector Delta EMV(-) 200.

The QqQ data were pre-processed with Agilent MassHunter Workstation Quantitative Analysis Software (B0700). Each metabolite was median normalized across all samples for proper comparisons, statistical analyses, and visualizations among metabolites. The statistical significance test was done by a two-tailed t-test with a significance threshold level of 0.05.

Proteomics: Cells were kept in control or media with $Serum^{HI}$ for six days. Cell were lysed and lysosomes were isolated as previously described [34] with anti-HA tag (Thermo Fisher #88836). Beads were washed twice with TBS-T and twice with PBS. The beads were resuspended in 50 mL of 0.1M ammonium bicarbonate buffer (pH~8). An overnight digestion with 1 μg sequencing grade, modified trypsin was carried out at 37 C with constant shaking in a Thermomixer. Digestion was stopped by acidification and peptides were desalted using SepPak C18 cartridges using manufacturer's protocol (Waters). Samples were completely dried using vacufuge. Resulting peptides were dissolved in 8 mL of 0.1% formic acid/2% acetonitrile solution and 2 mL of the peptide solution were resolved on a nano-capillary reverse phase column (Acclaim PepMap C18, 2μm, 50 cm, ThermoScientific) using a 0.1% formic acid/2% acetonitrile (Buffer A)

and 0.1% formic acid/95% acetonitrile (Buffer B) gradient at 300 nL/min over a period of 180 min (2-25% buffer B in 110 min, 25-40% in 20 min, 40-90% in 5 min followed by holding at 90% buffer B for 10 min and requilibration with Buffer A for 30 min). Eluent was directly introduced into Q exactive HF mass spectrometer (Thermo Scientific, San Jose CA) using an EasySpray source. MS1 scans were acquired at 60K resolution (AGC target=3x106; max IT=50 ms). Data-dependent collision induced dissociation MS/MS spectra were acquired using Top speed method (3 seconds) following each MS1 scan (NCE ~28%; 15K resolution; AGC target 1x105; max IT 45 ms).

Proteins were identified by searching the MS/MS data against UniProt H Sapiens database (20331 entries; downloaded on 12/04/2018) using Proteome Discoverer (v2.1, Thermo Scientific). Search parameters included MS1 mass tolerance of 10 ppm and fragment tolerance of 0.2 Da; two missed cleavages were allowed; carbamidimethylation of cysteine was considered fixed modification and oxidation of methionine, deamidation of asparagine and glutamine were considered as potential modifications. False discovery rate (FDR) was determined using Percolator and proteins/peptides with a FDR of ≤1% were retained for further analysis. Samples were normalized to the unbound fraction and relative peptide spectral matches were compared between control and Serum^{HI}.

Statistical Analysis: Statistical analysis was calculated by student's t-test, one-way, or two-way anova. Error bars represent the standard error of the mean.

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Table 2.1 qPCR and cloning primers

Gene	Primer
PRKN shRNA Fw	CCGGGCTTAGACTGTTTCCACTTATCTCGAGATAAGTG GAAACAGTCTAAGCTTTTT
PRKN shRNA 1 Rv	AATTAAAAAGCTTAGACTGTTTCCACTTATCTCGAGATA AGTGGAAACAGTCTAAGC
PRKN shRNA 2 Fw	CCGGGGAATGTAAAGAAGCGTACCACTCGAGTGGTAC GCTTCTTTACATTCCTTTTT
PRKN shRNA 2 Rv	AATTAAAAAGGAATGTAAAGAAGCGTACCACTCGAGTG GTACGCTTCTTTACATTCC
Lysozyme Fw	ATGGAATGGCTGGCTACTATGGAG
Lysozyme Rv	CTCACCACCCTCTTTGCACATTG
Cryptidins Fw	AGGAGCAGCCAGGAGAAG
Cryptidins Rv	ATGTTCAGCGACAGCAGAG
MMP7 Fw	CAGACTTACCTCGGATCGTAGTGG
MMP7 Rv	GTTCACTCCTGCGTCCTCACC
TFEB shRNA 1 Fw	CCGGGTCCGAGACCTATGGGAACAACTGCAGTT GTTCCCATAGGTCTCGGACTTTTT
TFEB shRNA 1 Rv	AATTAAAAAGTCCGAGACCTATGGGAACAACTGC AGTTGTTCCCATAGGTCTCGGAC
TFEB shRNA 2 Fw	CCGGCCTCTGTGGATTACATCCGGAGGATCTGC AGATCCTCCGGATGTAATCCACAGAGGTTTTT
TFEB shRNA 2 Rv	AATTAAAAACCTCTGTGGATTACATCCGGAGGATCTGCA GATCCTCCGGATGTAATCCACAGAGG
TFEB gRNA 1 Fw	CACCGATTGGGAGCACTGTTGCCAG
TFEB gRNA 1 Rv	AAACCTGGCAACAGTGCTCCCAATC
TFEB gRNA 2 Fw	CACCGGGACGACTCACTGCTACCGC
TFEB gRNA 2 Rv	AAACGCGGTAGCAGTGAGTCGTCCC

Cxcl2 Fw	TCCAGGTCAGTTAGCCTTGC
Cxcl2 Rv	CGGTCAAAAAGTTTGCCTTG
Il4 Fw	GGT CTC AAC CCC CAG CTA GT
Il4 Rv	GCC GAT GAT CTC TCT CAA GTG AT
Il6 Fw	ACCAGAGGAAATTTTCAATAGGC
Il6 Rv	TGATGCACTTGCAGAAAACA
Il1B Fw	AAGAGCTTCAGGCAGGCAGTATCA
Il1B Rv	TGCAGCTGTCTAGGAACGTCA
TNFa Fw	AGGGTCTGGGCCATAGAACT
TNFa Rv	CCACCACGCTCTTCTGTCTAC
Il10 Fw	AGACACCTTGGTCTTGGAGC
Il10 Rv	TTTGAATTCCCTGGGTGAGA
B220 Fw	TTCAGAAGCTGAACGTTGCACA
B220 Rv	TCTTCAGGAACCCCATGGTCTG
Il17 Fw	TGAGCTTCCCAGATCACAGA
Il17 Rv	TCCAGAAGGCCCTCAGACTA
Cdllb Fw	ATGGACGCTGATGGCAATACC
Cdllb Rv	TCCCCATTCACGTCTCCCA
Cd4 Fw	TCCTAGCTGTCACTCAAGGGA
CD4 Rv	TCAGAGAACTTCCAGGTGAAGA
Ym1 Fw	CACCATGGCCAAGCTCATTCTTGT
Ym1 Rv	TATTGGCCTGTCCTTAGCCCAACT

Cdllc Fw	CTGGATAGCCTTTCTTCTGCTG
Cdllc Rv	GCACACTGTGTCCGAACTCA
SAA4 Fw	CTCTGTTCTTTGTTCCTGGGAG
SAA4 Rv	CTAGGTTGTCCCGATAGGCTC
PINK1 shRNA 1 Fw	CCGGGAAATCTTCGGGCTTGTCAATCTCGAGATTGA CAAGCCCGAAGATTTCTTTTT
PINK1 shRNA 1 Rv	AATTAAAAAGAAATCTTCGGGCTTGTCAATCTCGAGA TTGACAAGCCCGAAGATTTC
PINK1 shRNA 2 Fw	CCGGGCCGCAAATGTGCTTCATCTACTCGAGTAGAT GAAGCACATTTGCGGCTTTTT
PINK1 shRNA 2 RV	AATTAAAAAGCCGCAAATGTGCTTCATCTACTCGAGT AGATGAAGCACATTTGCGGC
PINK1 Fw	CATGCCTACATTGCCCCAGA
PINK1 Rv	TGACTGCTCCATACTCCCCA

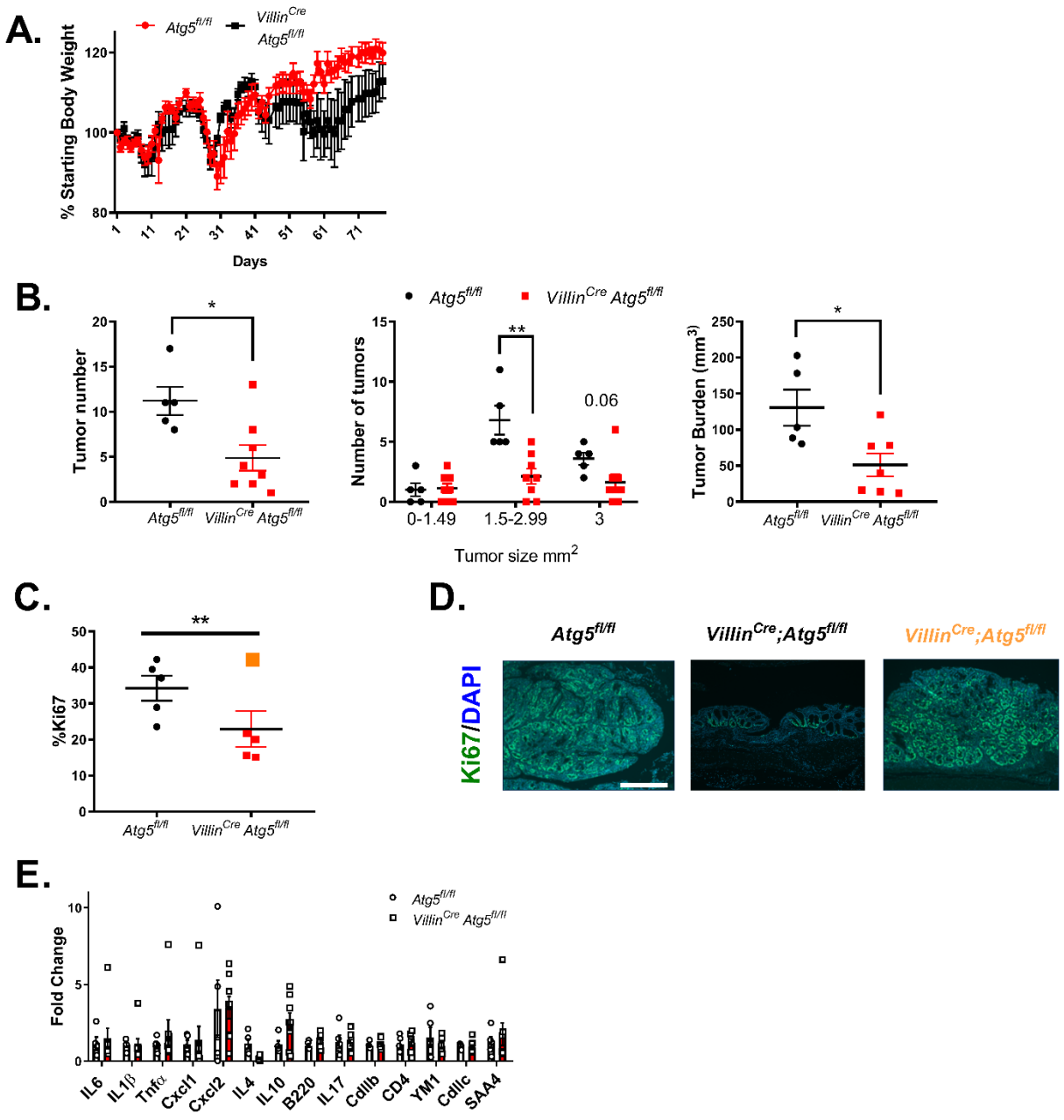

Figure 2.1 Epithelial loss of autophagy inhibits tumor growth in colitis associated cancer model. **A.** Body weights, **B**. tumor number, size, and burden, **C**. quantification of Ki67, (non-significant large tumor denoted in orange) **D.** images of Ki67 staining and **E.** qPCR analysis of cytokines and chemokines following AOM/DSS in colon specific *Atg5$^{fl/fl}$* and *Villin^{Cre}; Atg5$^{fl/fl}$* mice on AOM/DSS * p <0.05, ** p <0.01. Scale bar 200μm Error bars represent standard error of the mean (SEM).

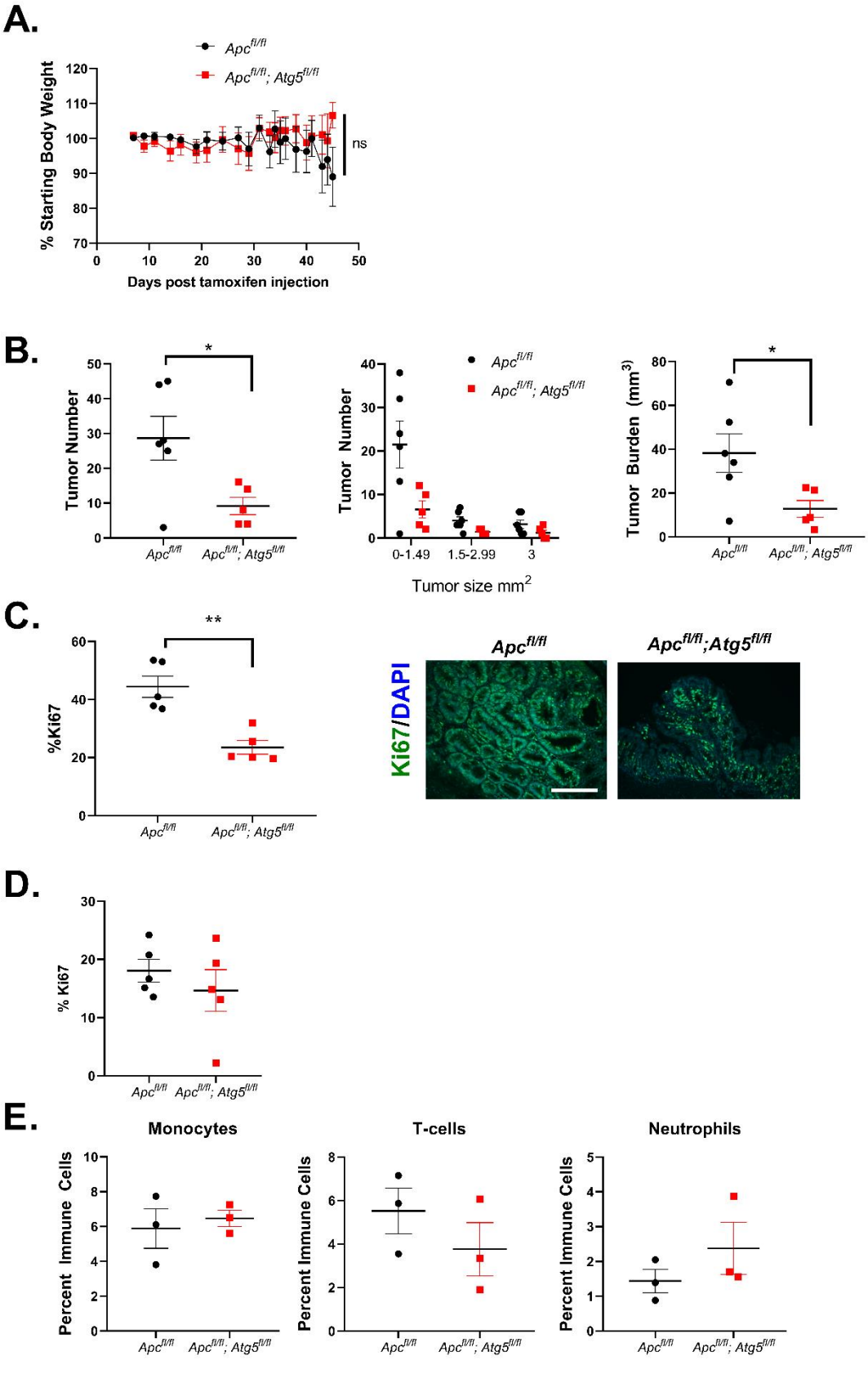
A.
Apc fl/fl
Apc fl/fl; Atg5 fl/fl
% Starting Body Weight
ns
Days post tamoxifen injection
B.
Tumor Number
Tumor Number
Tumor size mm2
0-1.49
1.5-2.99
3
Tumor Burden (mm3)
C.
%Ki67
Apc fl/fl
Apc fl/fl;Atg5 fl/fl
Ki67/DAPI
D.
% Ki67
E.
Monocytes
T-cells
Neutrophils
Percent Immune Cells

Figure 2.2 Epithelial loss of autophagy inhibits tumor growth in a sporadic colon cancer model. **A.** Body weights, **B.** tumor number, size, and burden, **C.** quantification and images of Ki67 stained tumor tissue, **D.** quantification of Ki67 in normal tissue and **E.** flow cytometry of immune cells in *Cdx2-ER^{T2}*Cre;*Apc$^{fl/fl}$*, and *Cdx2-ER^{T2}*Cre;*Apc$^{fl/fl}$*;*Atg5$^{fl/fl}$* mice. Tumors were assessed at 6-weeks following tamoxifen treatment, where flow cytometry was assessed at 2-weeks following tamoxifen treatment. * $p < 0.05$, ** $p < 0.01$. Scale bar 200μm. Error bars represent SEM.

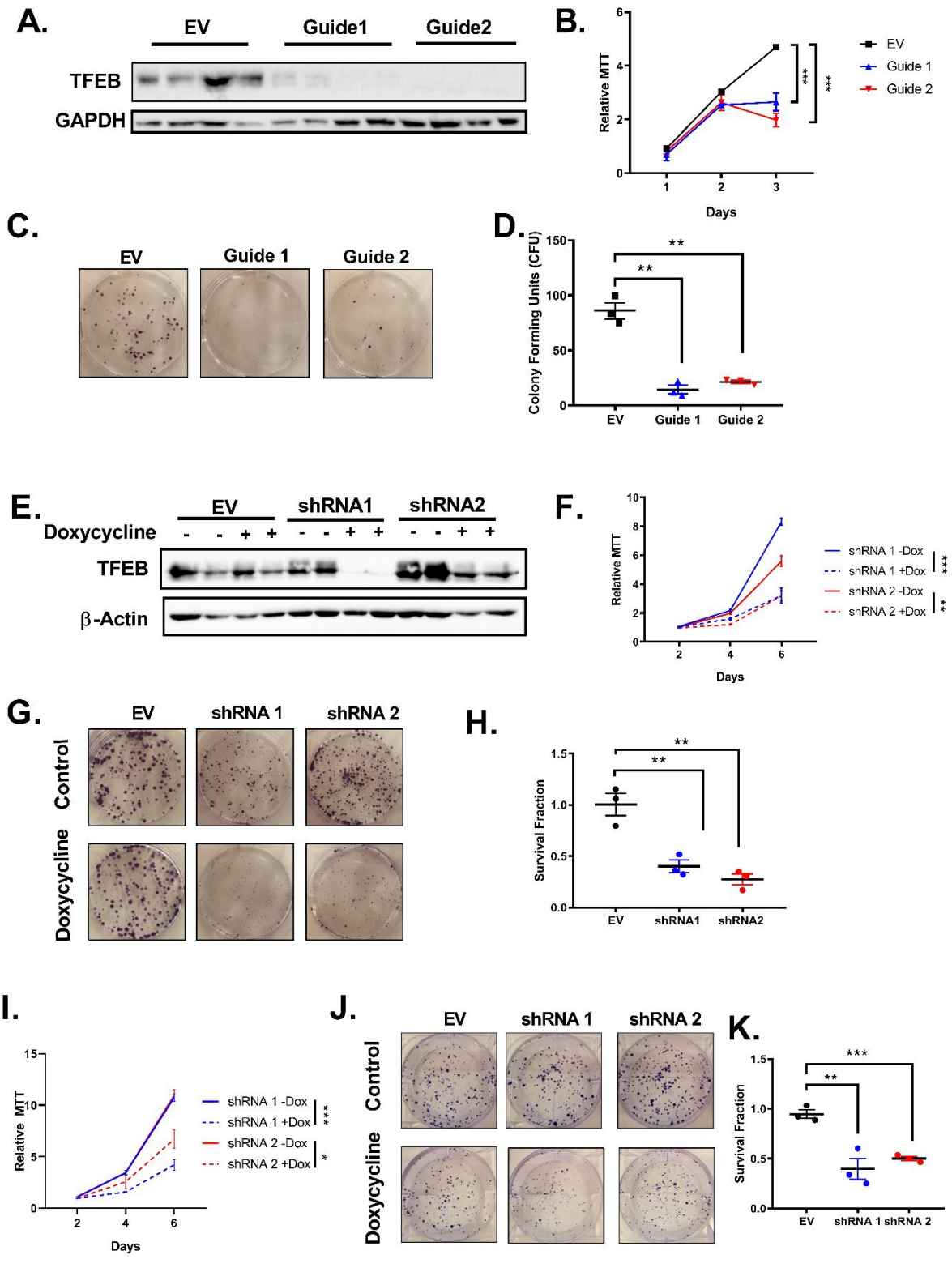
A.
EV
Guide1
Guide2
TFEB
GAPDH
B.
Relative MTT
Days
EV
Guide 1
Guide 2
C.
EV
Guide 1
Guide 2
D.
Colony Forming Units (CFU)
EV
Guide 1
Guide 2
E.
EV
shRNA1
shRNA2
Doxycycline
TFEB
β-Actin
F.
Relative MTT
Days
shRNA 1 -Dox
shRNA 1 +Dox
shRNA 2 -Dox
shRNA 2 +Dox
G.
EV
shRNA 1
shRNA 2
Control
Doxycycline
H.
Survival Fraction
EV
shRNA1
shRNA2
I.
Relative MTT
Days
shRNA 1 -Dox
shRNA 1 +Dox
shRNA 2 -Dox
shRNA 2 +Dox
J.
EV
shRNA 1
shRNA 2
Control
Doxycycline
K.
Survival Fraction
EV
shRNA 1
shRNA 2

Figure 2.3 Cell-autonomous inhibition of autophagy inhibits cell growth. **A.** Western blot analysis, **B.** MTT assay, **C.** representative images of clonogenic assay and **D.** quantification by blinded observers in stable HCT116 expressing empty vector (EV) or two different gRNAs specific for *TFEB* (Guide 1 and Guide 2). **E.** Western blot analysis, **F.** MTT assay (EV not shown), **G.** representative images of clonogenic assay and **H.** quantification of clonogenics by blinded observers in doxycycline inducible shRNA specific for TFEB (shRNA 1 and shRNA 2) or EV in HCT116. **I.** MTT assay (EV not shown), **J.** representative images of clonogenic assay and **K.** quantification of clonogenics by blinded observers in doxycycline inducible shRNA specific for TFEB (shRNA 1 and shRNA 2) or EV in SW480. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Error bars represent SEM.

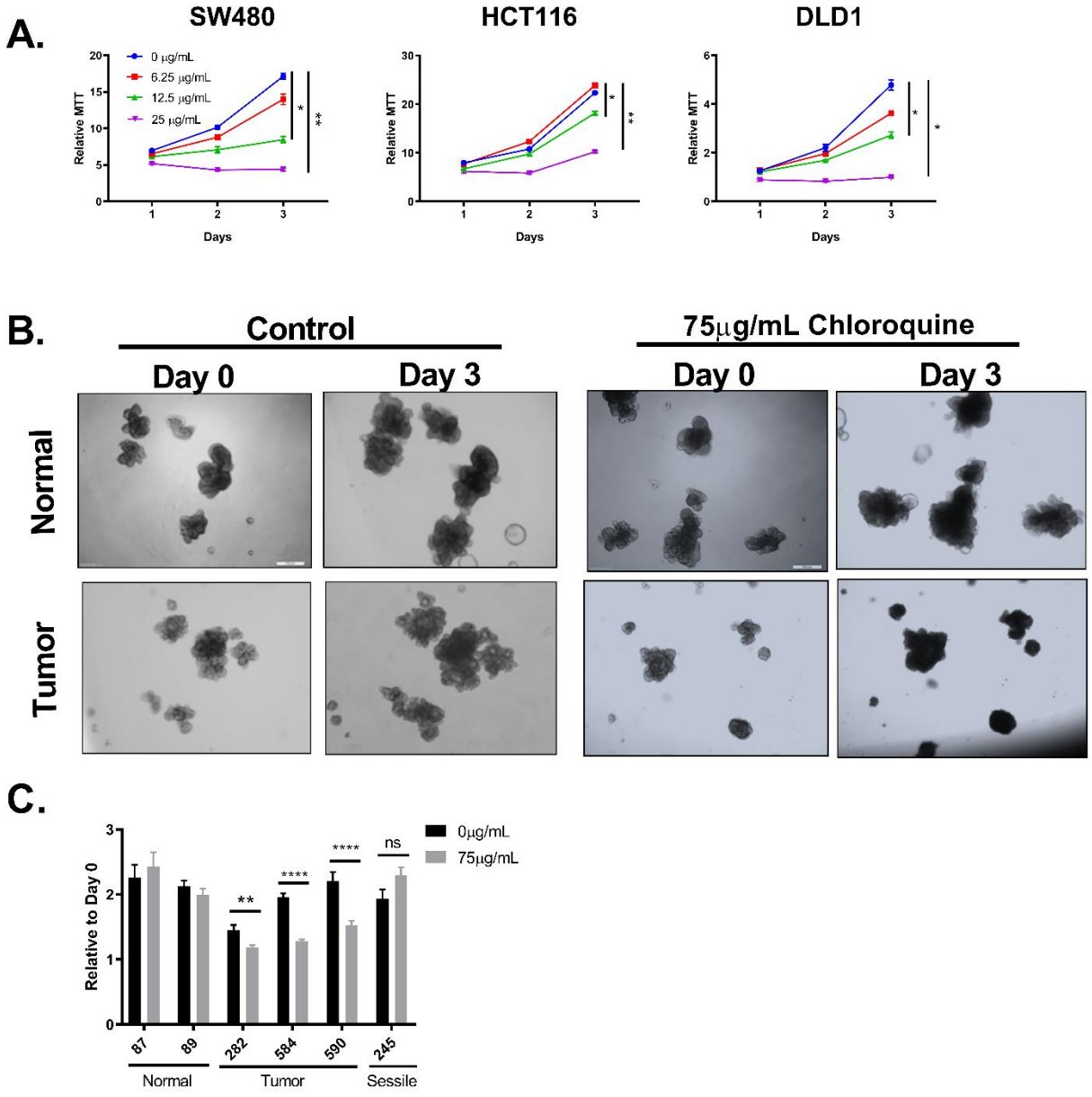

Figure 2.4 Pharmacological inhibition of autophagy inhibits CRC growth A. MTT assay in colon cancer-derived cell lines (SW480, HCT116 and DLD1) with chloroquine treatment. **B.** Representative images and **C.** growth quantification of normal and colon cancer patient derived enteroids treated with chloroquine for three days. Scale bar 500μm * p <0.05, ** p <0.01, **** p <0.0001. Error bars represent SEM.

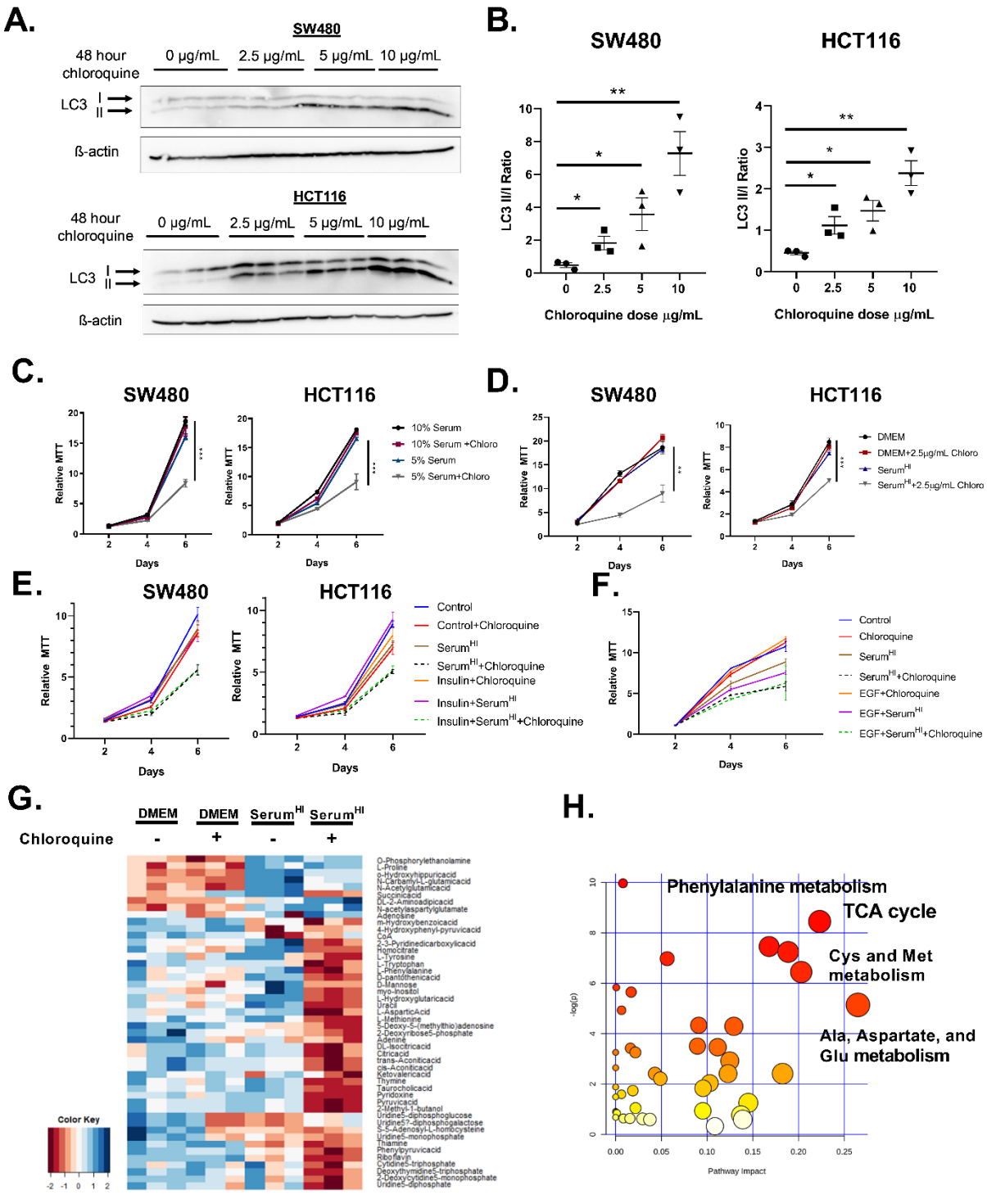
A.
SW480
48 hour chloroquine
0 µg/mL
2.5 µg/mL
5 µg/mL
10 µg/mL
LC3 I
LC3 II
ß-actin
HCT116
B.
SW480
HCT116
LC3 II/I Ratio
Chloroquine dose µg/mL
C.
SW480
HCT116
Relative MTT
Days
10% Serum
10% Serum +Chloro
5% Serum
5% Serum+Chloro
D.
SW480
HCT116
Relative MTT
Days
DMEM
DMEM+2.5µg/mL Chloro
Serum^HI
Serum^HI+2.5µg/mL Chloro
E.
SW480
HCT116
Relative MTT
Days
Control
Control+Chloroquine
Serum^HI
Serum^HI+Chloroquine
Insulin+Chloroquine
Insulin+Serum^HI
Insulin+Serum^HI+Chloroquine
F.
Relative MTT
Days
Control
Chloroquine
Serum^HI
Serum^HI+Chloroquine
EGF+Chloroquine
EGF+Serum^HI
EGF+Serum^HI+Chloroquine
G.
DMEM
DMEM
Serum^HI
Serum^HI
Chloroquine
- + - +
Color Key
H.
Phenylalanine metabolism
TCA cycle
Cys and Met metabolism
Ala, Aspartate, and Glu metabolism
-log(p)
Pathway Impact

Figure 2.5 Nutrient stress requires autophagy to maintain cell growth A. Western blot and, **B.** quantification of chloroquine dose to inhibit autophagy in HCT116 and SW480. **C.** MTT assay, cells were cultured in DMEM with 5% or 10% serum and in combination with chloroquine at 2.5µg/mL **D.** MTT assay, cells were cultured in DMEM with normal or $Serum^{HI}$ in combination with chloroquine at 2.5µg/mL. **E.** Supplementation of cells treated with $Serum^{HI}$ and chloroquine with insulin (10nM) in HCT116 and SW480 or, **F.** hEGF (50nM) in HCT116 **G.** Summary of Snapshot Metabolomics of SW480 cells with control or $Serum^{HI}$ or co-treated with vehicle or chloroquine. **H.** Metaboanalyst analysis of metabolites in $Serum^{HI}$ with chloroquine. * $p < 0.05$, ** $p < 0.01$. *** $p < 0.001$. Error bars represent SEM.

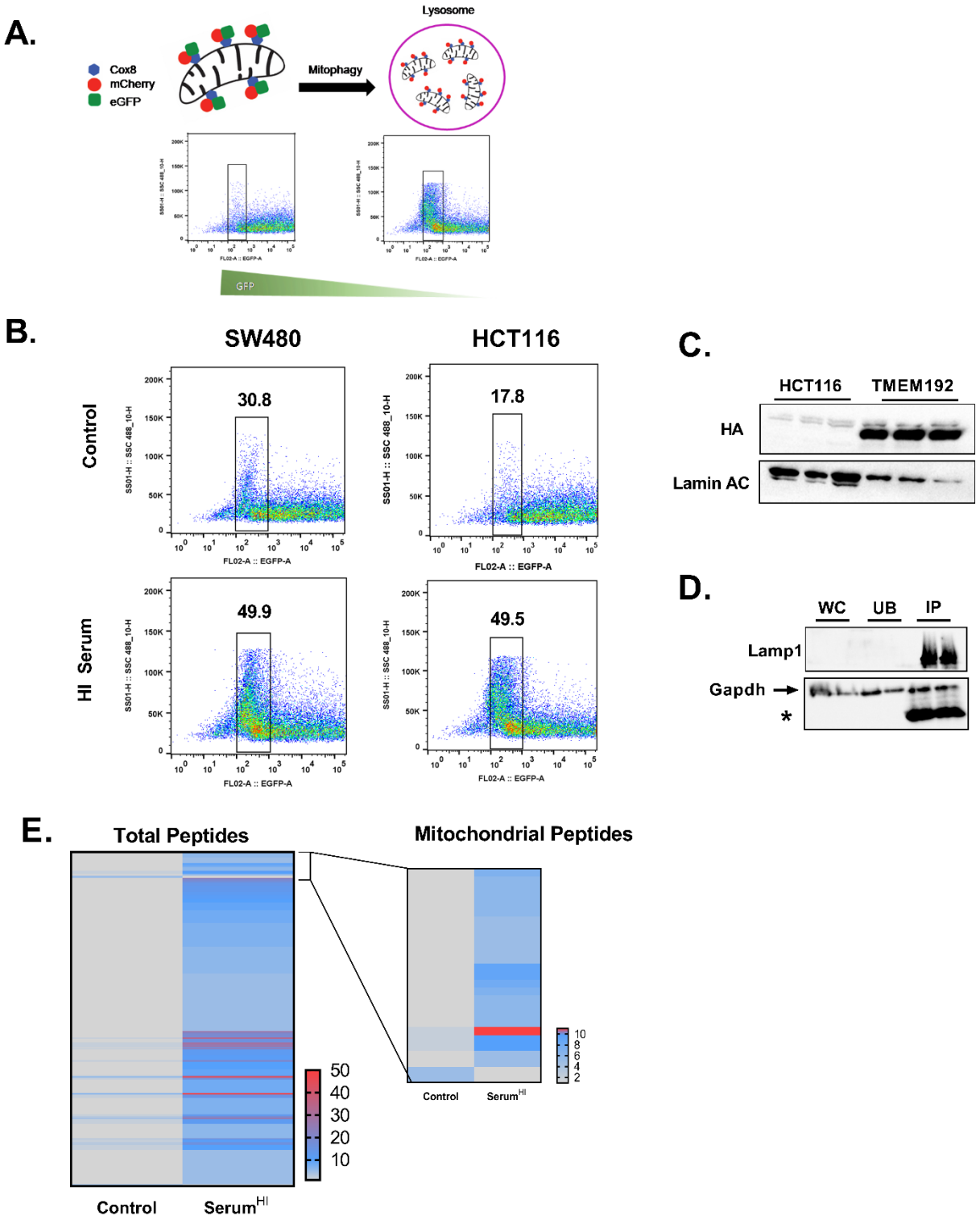
A.
Cox8
mCherry
eGFP
Mitophagy
Lysosome
GFP
B.
SW480
HCT116
Control
HI Serum
30.8
17.8
49.9
49.5
FL02-A :: EGFP-A
SS01-H :: SSC 488_10-H
C.
HCT116
TMEM192
HA
Lamin AC
D.
WC
UB
IP
Lamp1
Gapdh
*
E.
Total Peptides
Mitochondrial Peptides
Control
Serum^HI
50
40
30
20
10
10
8
6
4
2

Figure 2.6 CRC cells employ mitophagy under nutrient stress. **A.** Schematic of Cox8-mCherry-GFP flow cytometry. **B.** Flow cytometry analysis of mitophagy following two-day treatment with $Serum^{HI}$ in SW480 and HCT116 cells. **C.** Western blot confirmation of TMEM192-3xHA expressing HCT116 cells. **D.** Western blot of immunoprecipitation of TMEM192-3xHA cells in control or $Serum^{HI}$. WC- whole cell lysate, UB- unbound fraction, IP- bound sample. * represents degraded GAPDH product **E.** Relative change in peptide spectral matches to total and mitochondria specific proteins following treatment with $Serum^{HI}$.

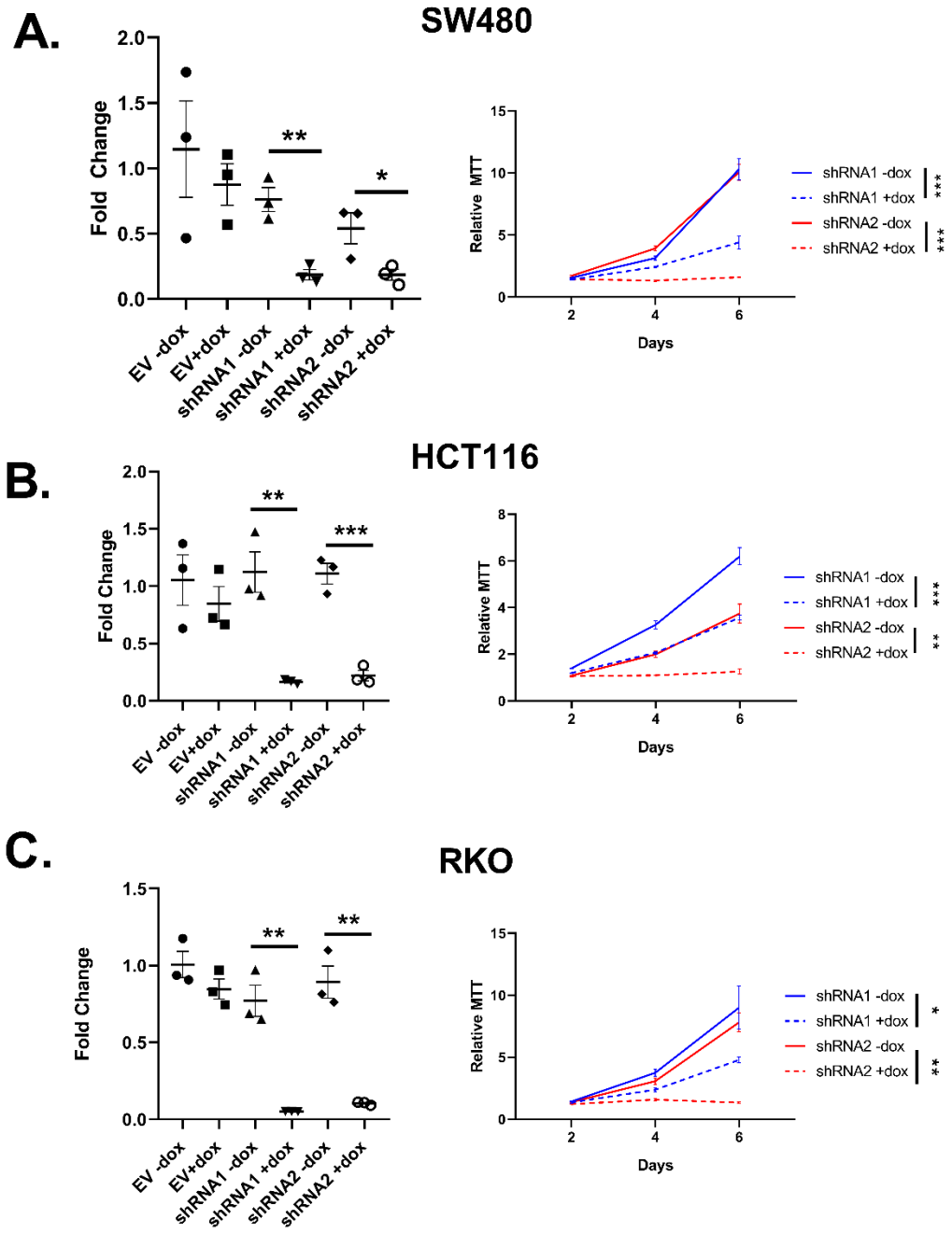

Figure 2.7 Mitophagy is necessary for CRC cell growth A. qPCR analysis of PINK1 shRNA knockdown and MTT assay (EV shown in Figure S7E) in SW480. **B.** qPCR analysis of PINK1 shRNA knockdown and MTT assay (EV shown in Figure S7E) in HCT116. **C.** qPCR analysis of PINK1 shRNA knockdown and MTT assay (EV shown in Figure S7E) in RKO. * p <0.05, ** p <0.01, *** p< 0.001. Error bars represent SEM.

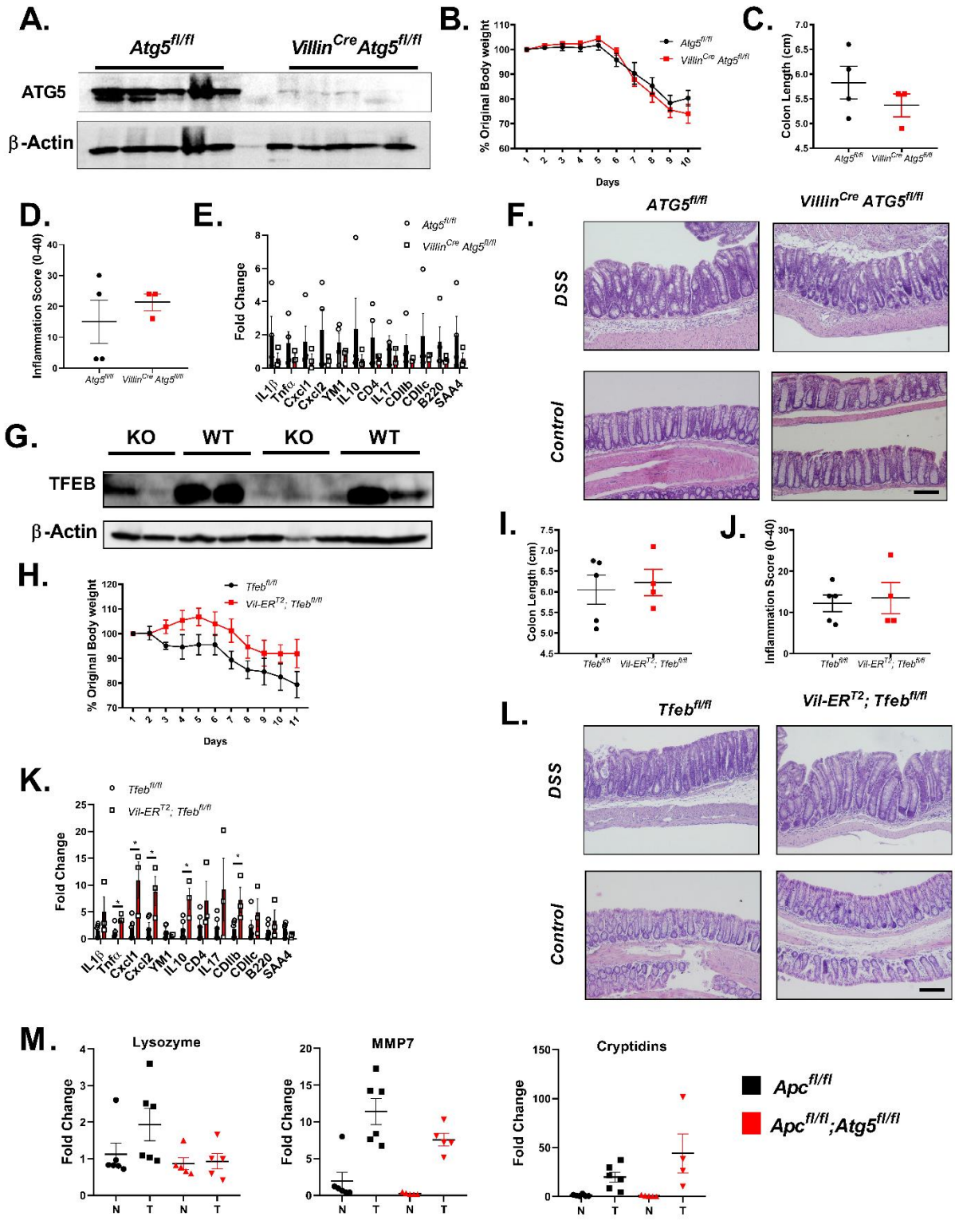
A.
Atg5fl/fl
VillinCre Atg5fl/fl
ATG5
β-Actin
B.
% Original Body weight
Days
Atg5fl/fl
VillinCre Atg5fl/fl
C.
Colon Length (cm)
D.
Inflammation Score (0-40)
E.
Fold Change
IL1β
Tnfα
Cxcl1
Cxcl2
YM1
IL10
CD4
IL17
CDIIb
CDIIc
B220
SAA4
F.
ATG5fl/fl
VillinCre ATG5fl/fl
DSS
Control
G.
KO
WT
KO
WT
TFEB
β-Actin
H.
% Original Body weight
Days
Tfebfl/fl
Vil-ERT2; Tfebfl/fl
I.
Colon Length (cm)
J.
Inflammation Score (0-40)
K.
Fold Change
L.
Tfebfl/fl
Vil-ERT2; Tfebfl/fl
DSS
Control
M.
Lysozyme
MMP7
Cryptidins
Fold Change
N
T
Apcfl/fl
Apcfl/fl;Atg5fl/fl

Figure S2.1 Autophagy loss does not impact acute colitis or Paneth cells in sporadic CRC. A. Western blot of *Atg5*$^{fl/fl}$ and *Villin*^{Cre}*; Atg5*$^{fl/fl}$ mice. **B.** Weights, **C.** colon length, and **D.** inflammation score of *Atg5*$^{fl/fl}$ and *Villin*^{Cre}*; Atg5*$^{fl/fl}$ mice following 7-day DSS with 3 day recovery. **E.** Panel of cytokines and chemokines following DSS in *Atg5*$^{fl/fl}$ and *Villin*^{Cre}*; Atg5*$^{fl/fl}$*.* **F.** H&E staining of control versus DSS treated mice. **G.** Western blot of *Tfeb*$^{fl/fl}$ and *Vil-ER*^{T2} mice. **H.** Weights, **I.** colon length, and **J.** inflammation score of *Tfeb*$^{fl/fl}$ and *Vil-ER*^{T2}*; Tfeb*$^{fl/fl}$ mice following 7-day DSS with 3 day recovery. **K.** Panel of cytokines and chemokines following DSS in *Tfeb*$^{fl/fl}$ and *Vil-ER*^{T2}*.* **L.** H&E staining of control versus DSS treated mice. **M.** qPCR analysis of Paneth cell markers in *Cdx2-ER*^{T2}Cre;*Apc*$^{fl/fl}$;*Atg5*$^{fl/fl}$ following 6 weeks of tumor formation. N-normal tissue, T- tumor tissue. *p <0.05. Scale bar 200μm. Error bars represent SEM

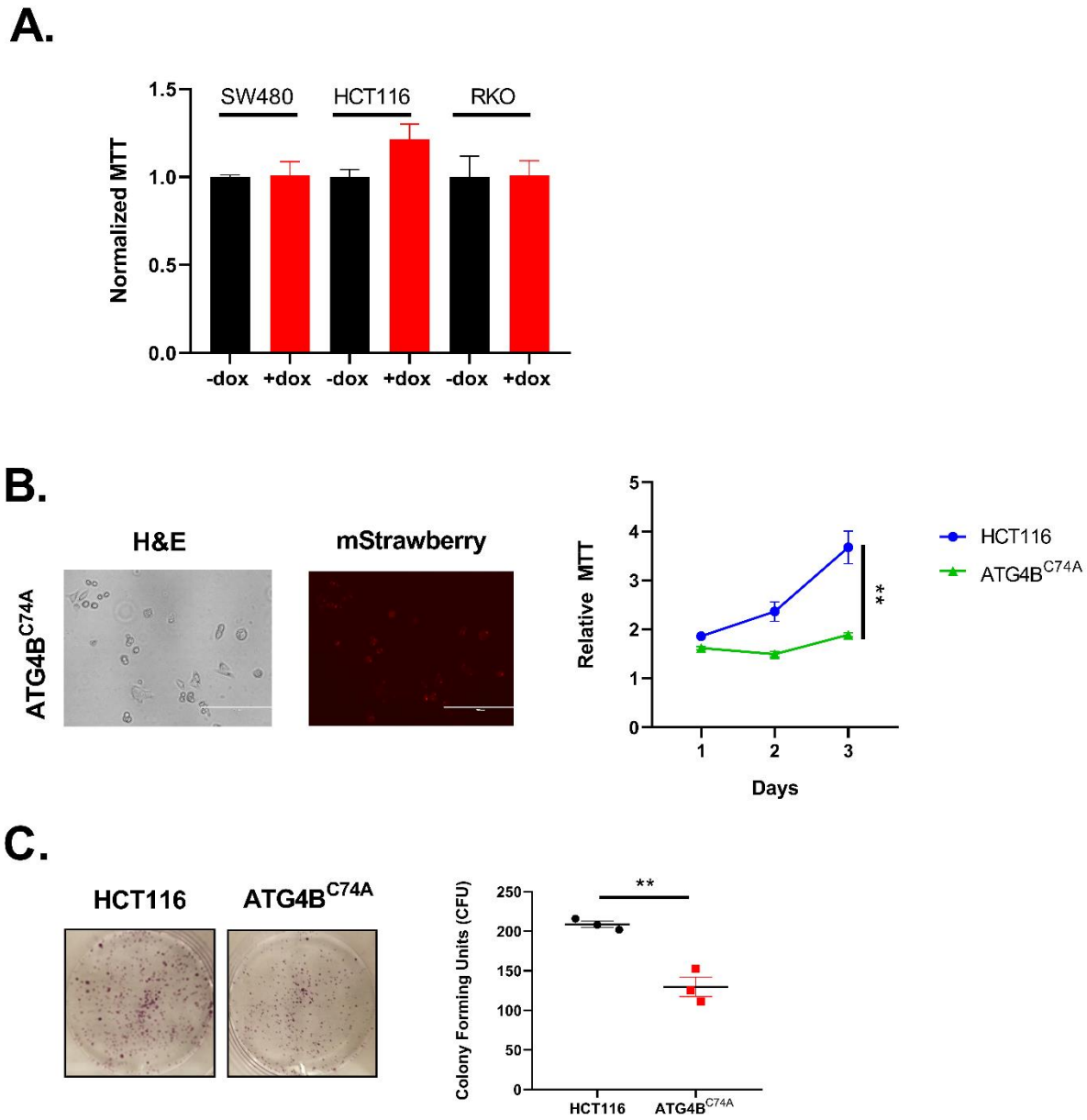

Figure S2.2 ATG4B inhibition reduces CRC growth. A. Analysis of empty vector constructs for doxycycline inducible shRNA constructs treated with doxycycline for 6 days. **B.** Image of mStrawberry expressing HCT116 ATG4B^{C74A} expressing cells and MTT assay of HCT116 ATG4B^{C74A} mutant. **B.** Representative image and quantification of clonogenic assay for ATG4B^{C74A} HCT116 cells. Scale bar 200µm. ** p<0.01. Error bars represent SEM.

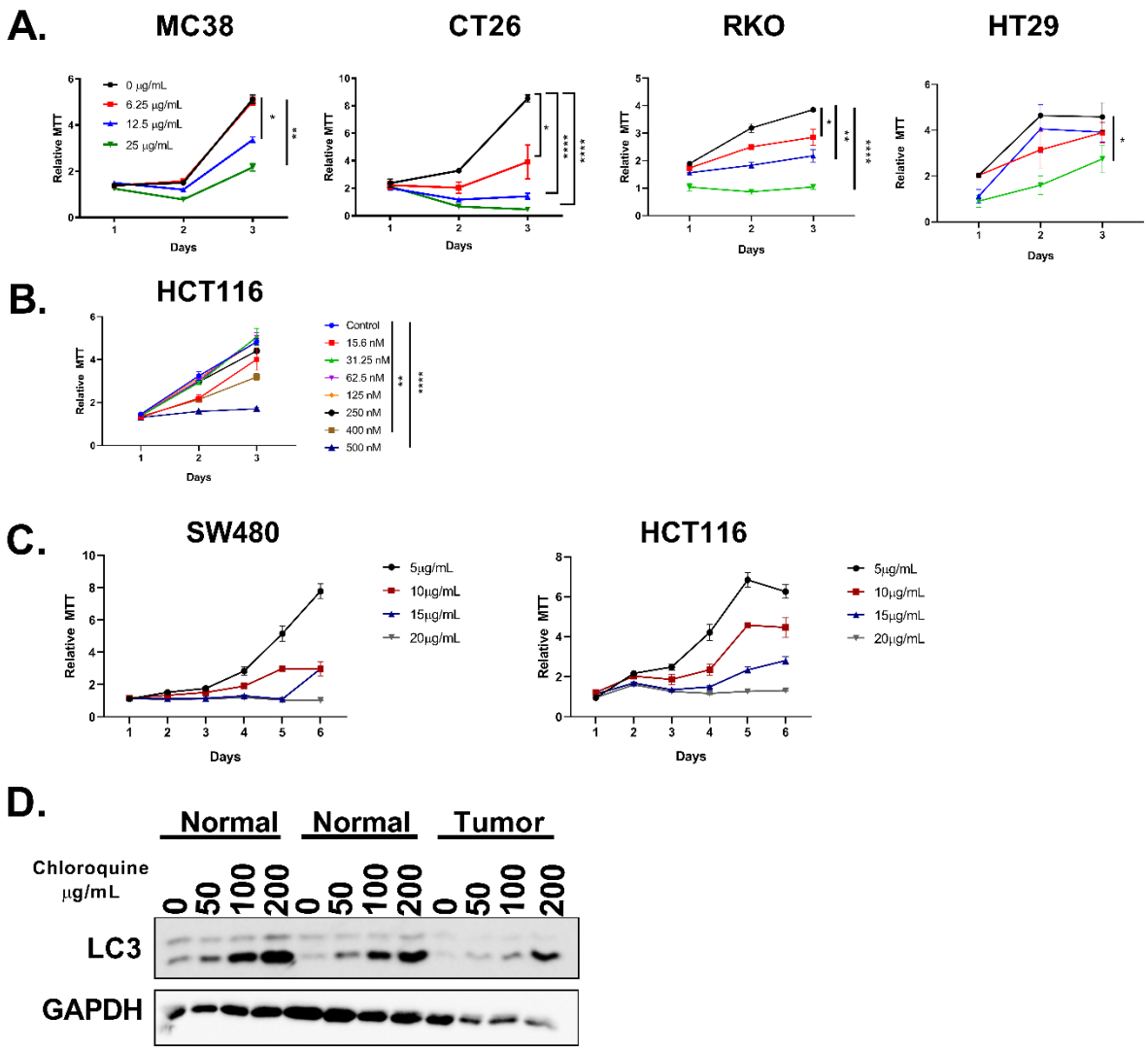

Figure S2.3 CRC cells are sensitive to pharmacological autophagy inhibition. A. MTT assay upon treatment with chloroquine in MC38, HT29, CT26, and RKO. **B.** MTT assay upon treatment with SBI-0206965 in HCT116 cells. **C.** MTT assay of HCT116 and SW480 cells treated for 3-days with chloroquine and measured for 3-days after removal of chloroquine. **D.** Western blot of LC3 expression in patient derived enteroids. * $p<0.05$, ** $p<0.01$, **** $p<0.0001$. Error bars represent SEM.

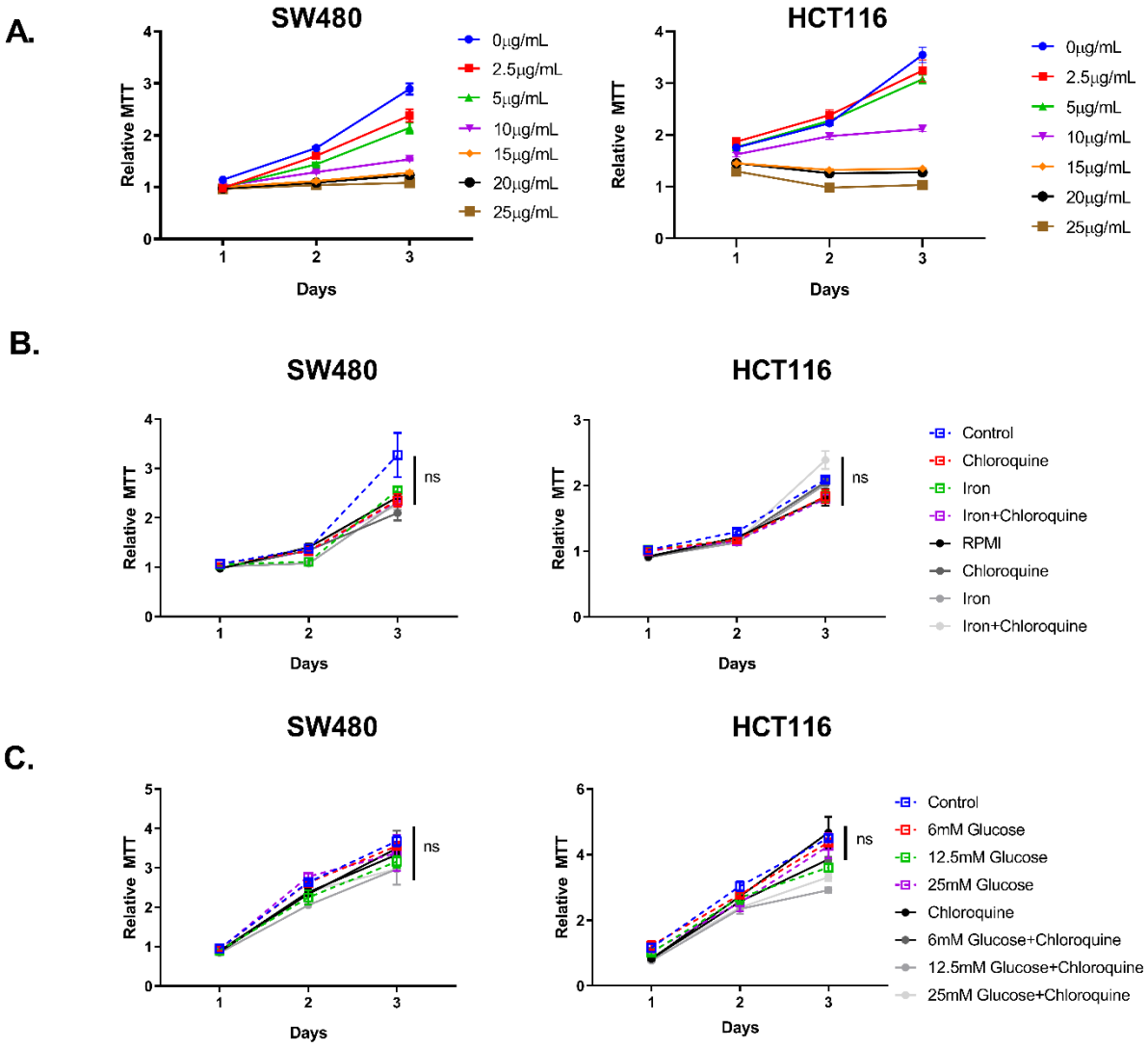

Figure S2.4 Nutrient challenge of iron or glucose loss with autophagy inhibition does not slow cancer cell growth. A. MTT dose response of chloroquine treatment in SW480 and HCT116 cells. **B.** MTT in iron deplete media and rescue with iron. **C.** MTT in glucose free media and rescue with glucose. Error bars represent SEM.

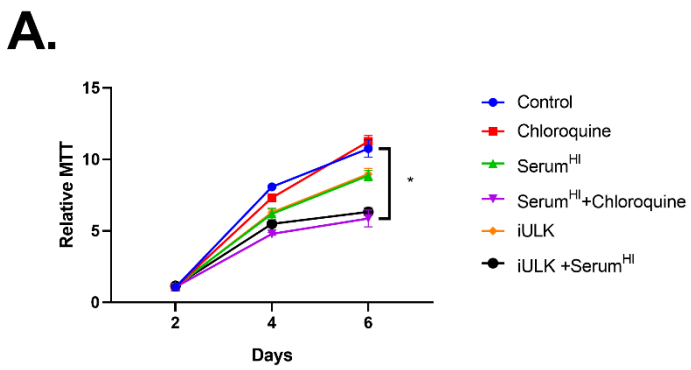

Figure S2.5 ULK inhibition stunts CRC growth. A. MTT of SBI-0206965 (iULK) treatment with Serum^{HI}. * $p < 0.05$. Error bars represent SEM.

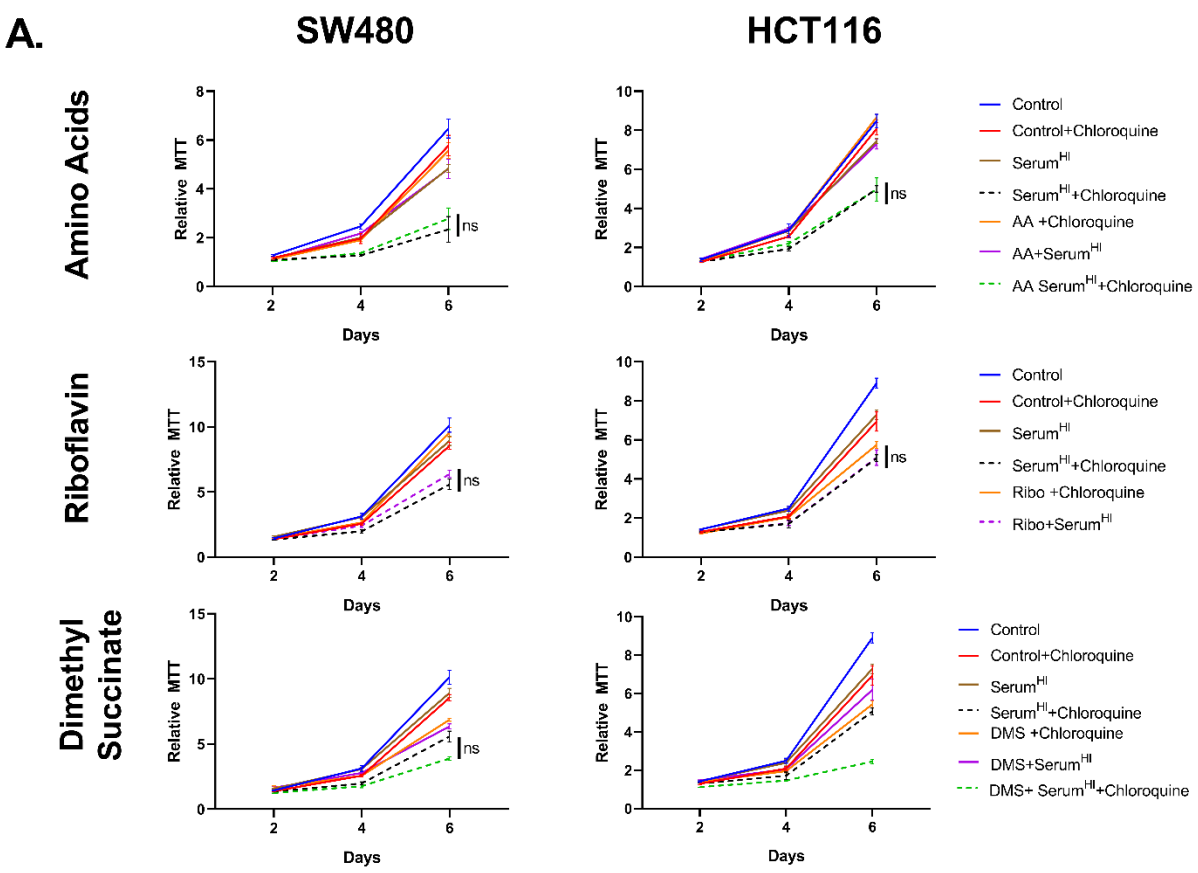

Figure S2.6 Metabolite modulation under Serum^{HI} and chloroquine does not rescue growth A. MTT assay of rescue with amino acids, riboflavin, and dimethyl succinate following treatment with Serum^{HI} with chloroquine. Error bars represent SEM.

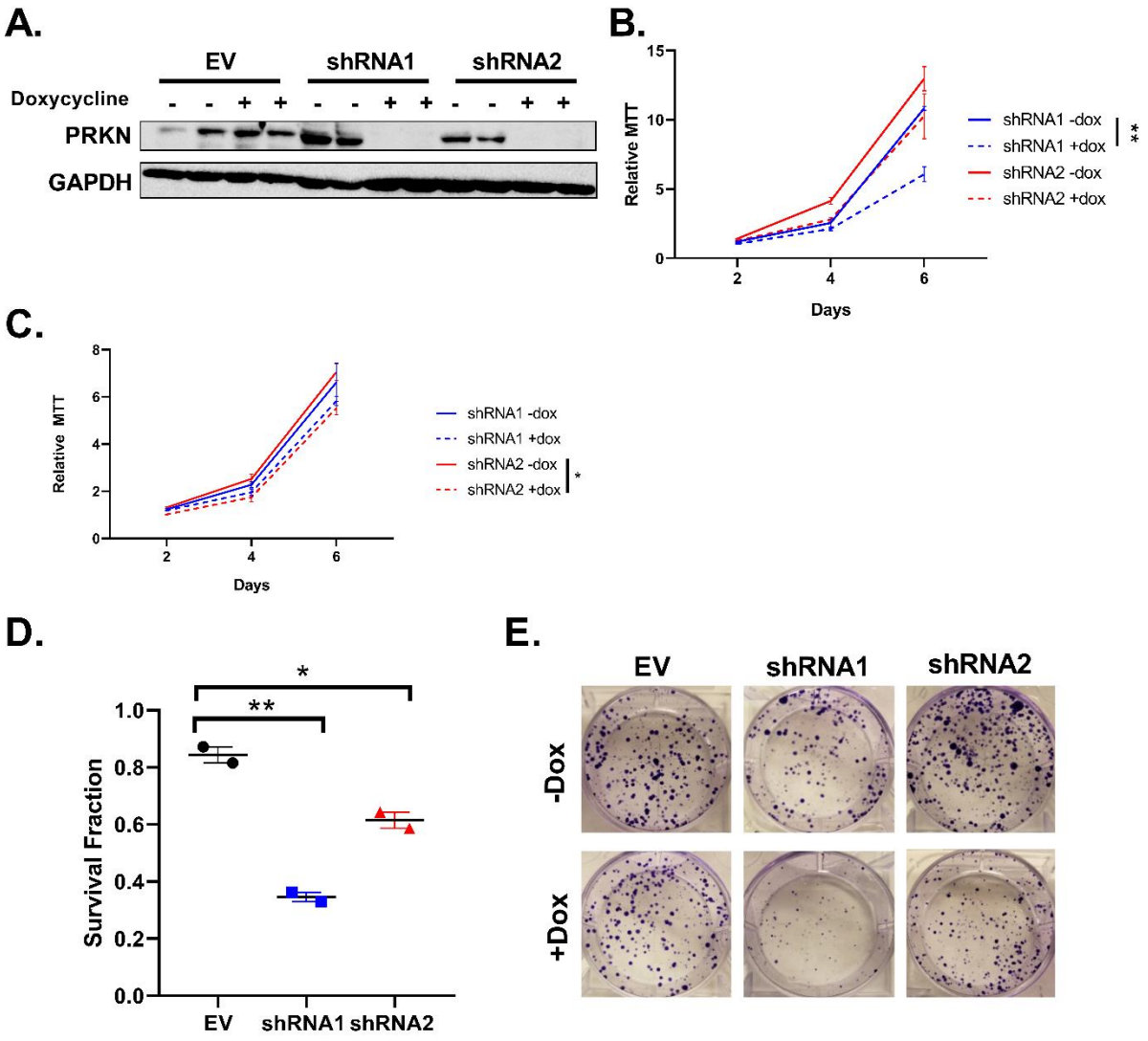

Figure S2.7 Mitophagy is necessary for CRC cell growth. A. Western blot analysis of PRKN knockdown in HCT116. **B.** MTT analysis of HCT116 PRKN knockdown (EV not shown) **C.** MTT analysis of RKO PRKN knockdown (EV not shown). **D.** Quantification of clonogenics by blinded observers in doxycycline inducible shRNA for PRKN in HCT116, and **E.** representative image of clonogenic assay. * p< 0.05, **p <0.01. Error bars represent SEM

Chapter 3

Single Cell Sequencing Reveals Epithelial Sub-Type Activation of Autophagy.

Abstract

The tumor environment is comprised of a heterogeneous mix of epithelium, fibroblasts, and immune cells that each contribute to tumor growth. Understanding cell specific signals in colon cancer will provide information to design targeted therapies for treatment. It is known in inflammatory bowel disease that Paneth cells are uniquely impacted by autophagy. While it is known that autophagy can modulate tumor growth, the cell type specificity of autophagy in colon cancer is not known. Bulk tissue RNA-sequencing can mechanistically be informative, but the cell types these transcriptional changes arise from are unclear. Here I utilize Seq-Well, a low-cost platform for single-cell transcriptome analysis to investigate the complex molecular role of autophagy in colon tumor cells. Single-cell RNA sequencing of a sporadic tumor model harboring *Apc, p53,* and *Kras*^{G12D} mutations uncovered an epithelial sub-type specificity of autophagy. Specifically, enterocytes express elevated levels of autophagy activating genes compared to goblet and enteroendocrine cells. This work reveals cell type specificity of autophagy activation in colon tumor development. Extensive work to investigate cell type changes in autophagy during tumor progression will guide the development of therapies that target specific cell types to better treat patients with cancer.

Introduction

RNA-sequencing is a powerful technique allowing for the full transcriptomic analysis of tissue or cell samples. The Cancer Genome Atlas (TCGA) is a collaborative cancer program between the National Cancer Institute and the National Human Genome Research Institute. Over the past decade, TCGA has gathered genomic, transcriptomic, and protein data that provides clinicians and researchers with tools to better study and treat cancer. Next-generation sequencing methods are also used to determine gene signatures and mutation burden that confer with growth, progression, and metastasis [1, 2]. Next-generation sequencing used in these publicly available databases typically involves sequencing patient biopsies. The tissue includes a combination of epithelial cells, fibroblasts, tumor associated immune cells, and tumor stroma. Bulk sequencing techniques have uncovered inflammatory signatures that are utilized to identify immune changes in tumors [3-6]. Over the past decade, there has been an emergence of the importance of tumor cell heterogeneity and stromal contribution to tumor growth [7]. Recent literature in colon cancer underscores the importance of cross-talk between tumor cells and tumor infiltrating immune cells [8]. Unfortunately, bulk transcriptomics do not provide researchers with the tools to understand the impact of immune cell populations or different epithelial cells in the colon. The rapid development of single-cell resolution techniques affords researchers the ability to evaluate cell type specific changes.

Methods for capture of single-cell whole-transcriptome (scRNA-seq) data began to appear about a decade ago [9]. Single cell sequencing methods have developed to investigate genomic, and transcriptomic changes. For transcriptomic analysis, methods typically involve isolation of a single cell into a chamber in which reverse transcription and

whole transcriptome amplification are carried out. Below is a review of the most commonly available methods, Drop-Seq, 10x Genomics Chromium System, and Seq-Well for single-cell RNA-sequencing. One of the earliest available methods was the Fluidigm C1 system which was previously widely used. Unfortunately, the small cell number output limits this technique as new approaches have become available.

Drop-Seq

Single-cell sequencing became more available to general users after introduction of the Drop-seq system developed by the McCarroll lab in 2015 [10]. Cells are passed through a “co-flow” microfluidic device that combines the flow of an oil channel with two aqueous solutions to generate nanoliter-sized droplets. One of the aqueous solutions has a microscopic nucleotide coated bead. The beads are generated using split-pool oligo methods. Synthesis occurs from 5’ to 3’ where the 3’ end is available for binding of mRNA. Each bead contains four regions, (1) an identical sequence on each bead that is used for primer binding that is important during the PCR step that will be described later. (2) A unique “cell barcode”, (3) a unique molecular identifier (UMI) that is used to differentiate duplicate sequences from the PCR step, and (4) an identical region that is used to capture polyadenylated mRNA [10]. Barcoding allows the user to identify which reads come from an individual cell and following amplification, the original number of transcripts. The microfluidic system combines a bead, cell, and lysis solution. Once the cells are lysed, the droplets are broken and subjected to reverse transcription. Beads that contain cDNA are referred to as STAMPS (single-cell transcriptomes attached to microparticles). Once cDNA is generated, sequencing can be performed. For this method, paired-end

sequencing is required to capture the barcode information and transcript information. It is estimated that the cost for constructing the system to perform Drop-Seq in the lab is around $6,000. One of the major pitfalls to this system is the high cell input number due to a capture rate of around 12%. The fluidics system creates a high number of droplets containing only a cell or only a bead requiring large input amounts, an approach that is not optimal for small or precious samples. This method is great for individual labs as the materials are obtainable but the complex fluidics may cause difficulty in use.

10x genomics

The most recent fluidics method available is the 10x Genomics Chromium System [11]. The Chromium System works in a similar method to Drop-Seq wherein a single cell is captured in what is termed a gel bead in emulsion (GEM). This setup contains a bead and a cell in a droplet that contains the reverse transcription reagents in the solution. The bead is similarly coated in oligo sequences containing an identical primer sequence. A unique cell barcode, and a UMI followed by a poly (dT) region. Similar to Drop-Seq, the cells are passed through a microfluidics system and then matched with a bead and placed in oil that partitions them into individual cell, bead droplets. Cell capture efficiency is significantly improved and ranges around 50%. Unfortunately, the 10x Chromium System requires the purchase of the controller system which was introduced at $50,000. This limits the use to certain users. In addition to instrumentation, a single sample can range around $6,000 dollars making it double the cost of Drop-Seq. A benefit to this approach is the time from cell isolation to lysis. Immediately after capture in the GEM cell lysis begins. One chip of the 10x system can hold 8 samples, each with a maximum target of

10,000 cells per sample. Cost to run the system will vary depending on the institution, at the University of Michigan, the cost is approximately $1,800 before sequencing.

Seq-Well

One of the major pitfalls to utilizing fluid-based techniques is the high cell number required for input in to the system, as well as the requirement to pass potentially sensitive cells through a fluidics system. To address these issues, the Seq-well system was designed by the Shalek lab at MIT [12]. This system utilizes a micro-well based technique. Cells are plated on an array that contains about 86,000 sub-nanoliter wells. Only 10,000 cells are needed to load the array. Arrays are loaded via slowly pipetting cell solution over the arrays, therefore there is no need for a fluidic system. Cells via gravity fall in to the wells that also contain beads as described in the section above. Once cells are collected, the array is sealed with a polycarbonate membrane. This membrane is unique in that it allows for the diffusion of fluids for lysis and hybridization but traps RNA in the well. When the membrane is attached, cells are lysed on the array followed by a hybridization of the RNA to the beads. A particular benefit to this method is the availability to gain protein information in parallel with sequencing data. Before cells are loaded on to the array, proteins may be labelled with conjugated antibodies. Then, the array can be imaged and quantified for protein level changes and can be compared to the transcriptomic data. The beads are manually removed from the array and are put through a reverse transcription. Following reverse transcription, a single stranded DNA digestion is performed. A whole genome amplification is done and the sample is removed from the micro-beads and submitted for RNA sequencing. A second advantage to this system is the flexibility of use.

While functionalizing arrays requires some special equipment, arrays can be shipped to any location and the protocol then only requires the arrays, membranes, pipettes, and reagents. This is in comparison to the other systems described here which require specialized equipment to run.

The above methods each have unique benefits and pitfalls. When selecting a method, the cost, cell type, and efficiency that is desired can go in to choosing a system. Another key aspect is the data output from each platform. The number of transcripts and genes captured from each cell is essential in gathering enough information to determine differences between cell types. Each of these platforms are generally comparable for gene and transcript output. The general workflow of each assay is comparable with main differences occurring at the cell capture step and whether each step is automated or manually completed (**Figure 3.1**). The output and cost of each platform is outlined in **Table 3.1**.

When preparing samples for scRNA-seq it is important to consider how isolation methods and origin of tissue may impact cell signature. Immediately after excising tissue, transcriptional changes and degradation of RNA molecules occurs. Attention to how tissues are collected, dissociated, enriched, and processed for single cell capture is essential in reducing technical variation [13]. Following generation of a single cell dataset, analysis is a significant feature in identifying single-cell variance.

Analysis of scRNA-seq

One of the largest challenges to performing single-cell RNA analysis is parsing the data to understand the relationship between cell types and function. With the development of better techniques for generating data sets, bioinformaticians are creating new ways to perform analysis to visualize data from thousands of cells. Tools are available to detect changes between healthy and disease states and predict cell fate during development [14]. As of March 2019, around 385 tools for scRNA-seq analysis exist [15]. The challenge surrounding single cell analysis is the availability of different platforms and standardization of best practices. Below I discuss general processing steps involved in the pre-processing of raw reads, data analysis, and general considerations for scRNA-seq analysis.

Sequencing: To appropriately capture sequence from methods such as Drop-Seq and Seq-Well, paired-end sequencing must be performed. Read1 can be completed with a 26- base pair (bp) read. This 26bp read captures the UMI and cell barcode. A 50bp Read2 on the 3' end captures enough bp's that can be used to determine the transcript. It is important when sequencing to ensure that the average size of the products is greater than 420bp. This requirement prevents the potential reading of poly-A tail reads that would occur if sequences were shorter. While this sequencing depth was used for this dissertation, deeper sequencing can be performed to gather more information from individual samples.

Pre-processing: When sequencing with barcoded beads the reads are first organized by their UMI and cellular barcodes. Sequences are then filtered to remove any cell or molecular barcodes that are below a base quality threshold. This will prevent

matching any reads with the incorrect cell. SMART adapter sequences are trimmed that may be present at the 5’ end of the read so they are not present during sequence alignment to the genome of interest. While library preparation enriches for sequences with longer length, it is possible to obtain sequences with poly-A reads. Any reads with more than 6 sequential adenine bases are removed. The library is then aligned to the genome and annotated. Any reads that overlap with an exon are tagged and exported for analysis.

Data Analysis: Once the sequences are organized by cell and aligned to the genome, an initial quality check is performed. The quality check includes observing the number of genes per cell, number of transcripts per cell and mitochondrial genes [16]. Cells with high mitochondrial reads or low gene count can be filtered out. High mitochondrial reads may indicate that cellular mRNA has leaked out of a damaged membrane leaving behind only mtRNA. Cells high in gene counts may be indicative of doublets where two cells were captured on one bead. It is important to consider that some cell types may be highly proliferative or quiescent which will impact their counts. Downstream analysis can be variable based on the user approach and dataset. Analysis typically outputs cells in to clusters which groups cells based on the similarity of their gene expression. Differential expression between a group of cells and all of the remaining cells in the dataset can be used to determine the gene differences in the cluster of interest. Cell clustering will vary based on parameters set during analysis [17]. The tool used in this dissertation for analysis is Seurat, developed by the Satija lab at MIT [18]. Using Seurat, the resolution of clusters can be modified to define more or less clusters. This number will vary based on the small versus broad changes that researchers want to

determine in a dataset. With the rise of single-cell sequencing reference databases are being generated so that clusters can be annotated using a reference data set rather than *de novo* and determined by known markers [19]. However, due to experimental differences, using gene expression from the data is always an important approach. In order to reduce technical noise, spike-ins of RNA can be measured and analyzed to compensate for variation [20]. Considerations must be made when analyzing changes in cell types in differentiation or dynamic models. One approach is using the trajectory inference method. This process takes a snap-shot of data and interprets it as a path or trajectory [21]. An example of this application is determining the trajectory of sperm differentiation [22]. Analysis may also compare changes between treatment groups or disease states. With the emergence of new approaches of analysis there are unique and beneficial ways to analyze data. A review of some of these computational approaches and methods can be found here [23].

Single cell analysis in the colon

The colon is comprised of multiple cell types that play important roles in tissue homeostasis. The colon is a highly proliferative organ that constantly regenerates. Stem cells are the driving factor that supports this high tissue turnover. Colonic stem cells located in the base of the crypts are typically identified by Leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) positivity [24]. They differentiate to generate enteroendocrine, enterocytes, goblet cells, and the other diverse cell types of the colon that are discussed below.

Enteroendocrine

These cells control enzyme secretion, secrete peptides, and can control appetite. Subclasses of enteroendocrine cells exist based on their secretory products [25]. They function in chemosensing and nutrient sensing in the gut and can help to provide signals to the brain [25, 26]. Based on the diversity of sub-types of enteroendocrine cells, they can be identified by a wide range of markers with a main marker being claudin-4 [27, 28].

Goblet Cell

Goblet cells are the primary mucin forming cell of the colon. The secretion of mucins provides a protective barrier from the gut associated microbiota. These cells are marked by intestinal trefoil factor 3 (TFF3) [29]. Subtypes of mucinous cells exist that add complexity to the cell type [30].

Enterocytes

Enterocytes are a primary absorptive cell in the colon. Enterocytes are involved in processing antigens to present to the immune system. One of the major mechanisms to perform this function is through lysosomal degradation [31]. Enterocytes also play a role in nutrient and water absorption.

Differences in the cell types discussed above were examined in the small intestine using single cell sequencing [32]. These different cell types can be identified with single cell sequencing by known markers. Cells were grouped by Slc12a2, Arg2, TFF3 and Defa24 [32]. This study was performed in the small intestine and while the cell types are

similar in the large intestine it is important to consider that expression signatures may change.

Little work has been done to assess the transcriptome differences between colonic cell types, particularly in colon cancer. Sequencing of 34 colorectal cancer cell lines revealed differences in DNA copy number, mRNA, miRNA, and protein analysis between different patients [33]. The study provides information about a model system to study colon cancer. This system is useful in that you get a gene signature specific to epithelial cells. The caveat to this approach is that surrounding supportive cells including fibroblasts and immune cells are lost with line establishment. Studies have revealed the transcriptional changes between patient derived tissue and their matched cell lines [34]. It is important to note that over time, when serial passages are done on cell lines that the transcriptional landscape continues to change [34].

Understanding tumor cell of origin may help researchers to identify potential targets to treat colon cancer. In one study of two patient derived samples, normal and colon tissue from non-hereditary colon cancer was examined with bulk and single-cell whole exome sequencing. Analysis revealed sub-clonal populations with somatic mutations [35]. These observations were not available in bulk samples which may hide potential hits due to the multiplicity of cell types in the sample. It is important to know that these mutational calls were made on 25 single cells. Due to the heterogeneity of the colon, certain cell populations may be missed with this small sample pool.

As highlighted in Chapter 1, understanding the function of autophagy in different cell types can provide novel insight on the impact of autophagy in CRC. With bulk

sequencing, parsing out signals of autophagy is near to impossible. The single cell approach will allow researchers to understand the immune versus epithelial changes in autophagy as well as changes between different types of colon cancer cells. Here I utilize a sporadic model of colorectal cancer to explore changes in autophagy between colonic cell types. Colon cancer patients typically harbor mutations in *APC, p53,* and *KRAS.* I obtained a triple mutant mouse model that is colon specific (*Cdx2-CreER*^{T2}; *Apc*$^{fl/fl}$*; Tp53*$^{fl/fl}$*;Kras*^{G12D}) herein the 'TripleMut' to investigate sporadic colon cancer changes. When mutations are induced in this model there is a high level of dysplasia present and mice typically die within 10 days due to severe dehydration. The dysplasia causes a high influx of immune cells and hyper-proliferation of epithelial cells. I chose to utilize this model to investigate changes in the autophagy signature in different colonic cell types under this high mutational load that more closely recapitulates human mutational burden.

Results

TripleMut mice were induced with tamoxifen at 100mg/kg for 3-days. Due to the highly proliferative nature of this model the ratio of epithelial to immune cells would mask any immune cell populations and make it difficult to detect them via single-cell sequencing. I optimized a protocol to enrich for immune cells. I optimized enrichment of immune cells and validated using flow cytometry that I could extract viable, CD45+ immune cells (**Figure 3.2**). Isolated tissue was subjected to single-cell sequencing using the Seq-Well platform (**Figure 3.3**) [12].

Single cell sequencing revealed both immune and epithelial subtypes (**Figure 3.4**). Populations identified include fibroblasts, enteroendocrine, enterocytes, goblet cells, B-cells, macrophages, and red blood cells. When the cluster of absorptive cells was compared to the enteroendocrine cell types, an increased expression of Itgb4, Itga6, Hsp90ab1, Myc was observed (**Figure 3.5**). Increases in these autophagy genes correlate with activated autophagy [36-38]. Similarly, there is a decrease in expression of SQSTM1 which correlated with increased autophagy [39]. When comparing absorptive cells to goblet cells, there is a decrease in Birc5 suggesting an autophagy increase [40, 41].

When compared to bulk RNA-seq from proximal colon of TripleMut mice compared to controls, there is no difference observed in these autophagy markers (**Figure 3.5**). This highlights the observed changes that can be found with single cell sequencing that cannot be detected with bulk sequencing. However, changes in SQSTM1 can be found

compared to control suggesting an overall upregulation of autophagy in highly dysplastic mice bearing these mutations.

Discussion

Here I have begun the first investigation to understand the cell type specific modulations of autophagy in colon cancer. The TripleMut mice closely model the mutational burden seen in human CRC compared to models such as the Azoxymethane/Dextran Sulfate Sodium inflammation model. I wanted to investigate both immune and epithelial populations in the colon. Due to the highly proliferative nature of the TripleMut model, I performed an enrichment for immune cells. This protocol is a mixture of of mechanical dissociation, enzymatic digestion, and enrichment via centrifugation occur. While I have worked to optimize the shortest method possible, the isolation takes approximately 3 hours. This timing and cell stress of dissociation can have an impact on the gene signatures observed. For example, while Hsp90ab1 was significantly increased in the enterocyte population compared to the enteroendocrine cells it was highly expressed in all cell types. This is possibly due to an increase in cell stress [42].

Unfortunately for this study I did not account for the high amount of red blood cells that are present in the sample. The large population present may be due to the high ratio of these cells in the sample. Therefore, I further optimized the protocol to include a red blood cell lysis step which will enrich further for the cell type of interest. Removal may also increase capture of other immune cell types to add diversity and numbers to the comparisons. Additionally, the disadvantage to a well system based on gravity is differences in cell size. When loading the cells, the standard protocol is to wait approximately 10 minutes for cells to fall in to a well. For smaller cells this may take a longer period. Optimizing the protocol with this in mind is important for future experiments.

The lack of ability to call certain populations may be contributed to the differentiation process of cells in the colon. Intermediate cell types include oligomucous cells that differentiate to goblet cells, low granule enteroendocrine cells, and midcrypt columnar cells that differentiate in to villus columnar cells [43]. Performing analysis with a higher resolution may allow for the separation of more clusters that could define these intermediate populations. However, it would be best to perform the analysis with a larger cell pool to ensure positive identification in smaller populations. For example, analysis of a mouse dataset with over 90,000 cells could identify rare populations in 132 cell clusters [18].

The importance of being able to call intermediate populations is emphasized by the development of the Human Cell Atlas which aims to catalog the genomic and phenotypic landscape of every cell type in the body. The consortium hopes to define how these cells change under developmental and diseased states. Due to the sensitivity of identifying each cell type and during different stages of age and health it is important to utilize a method that reduces technical variability. Due to the automated and hands-off function of the 10x system it has been used to begin generating libraries for the Human Cell Atlas. Because multiple users will contribute to the database around the world, a highly functionalized system is useful. The pitfalls for Seq-Well and Drop-Seq are the user error that can be input.

The TripleMut model used here is a valuable tool for future research. For this particular experiment mice were induced at a high dose of tamoxifen and euthanized almost 10 days after. Mice die at this time point due to hyper-proliferation of the colon. This hyper-proliferative phenotype may impact the gene signature discussed here and

may not accurately model what is observed in CRC patients. I have identified a low dose of tamoxifen (single dose 25mg/kg) that allows mice to survive past 6-weeks and develop individual tumors rather than a highly dysplastic colon. In mice that have been induced with a low dose of tamoxifen, tumors can be collected at early, middle, and late stages of tumor development and single-cell changes can be observed within different cell types. An important comparison is also the progressive accumulation of mutations within the colon. The TripleMut mice should be simultaneously compared to mice with *Apc* only, or *Apc;p53* mutations to better understand how mutation load impacts development. While here I have primarily focused on autophagy there is a breadth of information that can be acquired from databases generated in these studies. Overall changes in immune markers can be investigated to better understand single-cell changes and mice can be challenged with different therapeutics to monitor response. Autophagy models such as the *Apc*$^{fl/fl}$*;Atg5*$^{fl/fl}$ model can be used to investigate how loss of autophagy impacts tumor epithelial cells.

To fully utilize scRNA-seq it is important to know the capabilities of the technique when approaching a new question. In cases where researchers may wish to identify new areas of focus or ask broad questions, performing a single round of 10x sequencing may be beneficial. Typically, a trained technician will be able to process the samples allowing for limited troubleshooting and ease of use. However, if a project is aimed at understanding timing or spatial changes in a disease or healthy state that simultaneously requires multiple samples, a platform where multiple samples can be run in parallel such as Seq-Well is more efficacious. Similarly, for the generation of larger datasets, platforms such as Drop-Seq and Seq-Well are more desirable due to reduced cost. The available

access to a 10x Chromium system, the fluidics for Drop-Seq, or reagents for Seq-Well will also impact the choice of platform.

Single-cell transcriptomics is a powerful tool that provides insight on a cell by cell basis that is not detectible through other methods. Limited information is available for scRNA-seq in healthy colon and in diseased states such as CRC. This work only begins to uncover differences between colonic cell types in tumor development. Single-cell sequencing of different models throughout tumor development will provide insight on changes in autophagy. In the future, single-cell sequencing should also be applied to patient samples to uncover information not detected in currently available models. Single cell resolution will provide in-depth information that can be used in research to improve our understanding of CRC biology.

Methods

Mice: Mice were housed in standard housing conditions and fed *ad libitum.* Both male and female mice were used. *Cdx2-CreER^{T2} Apc^{flox} p53^{flox} Kras^{G12D}* TripleMut mice were injected with three doses of 100mg/kg of tamoxifen. Nine days following the first injection, mice were euthanized using CO_2. Colons are removed and washed in phosphate buffered saline.

Tissue Isolation: Tissue is cut in to 1cm pieces and shook at 150 rotations per minute at 37ºC in 10mM EDTA. Following incubation, tissues are vortexed to release epithelial cells. The tissue is then minced in to 1mm pieces and placed in a collagenase solution (0.5mg/mL Collagenase type IV in RPMI) and shook at 250rpm at 37C. Tissues are then vortexed and collected for further isolation. The cell pellet is passed through a 100µM filter to remove large debris and then mixed in 40% percoll and laid over 70% percoll. The cells are spun and the middle, immune cell enriched layer is collected. Cells are then passed through a 40µM filter and are ready for array loading.

Flow Cytometry: Cells were isolated as described above. Cells were stained using CD45 Alexa Fluor 780, 1:200 (eBioscience), and CalceinAM (1:200). Analysis was performed on the MoFlo Astrios (Beckman Coulter).

RNA isolation and qPCR analysis: RNA was isolated using TRIzol chloroform extraction. RNA was reverse transcribed using MMLV reverse transcriptase (ThermoFisher). qPCR analysis was done using the listed primers (**Table 3.2**) and Radiant Green qPCR master mix (Alkali Scientific Inc.).

Array Functionalization: Arrays are functionalized in the following fashion. First, Polydimethylsiloxane is poured in to the array mold containing glass slides. Following heating at 70C for 2-hours arrays are removed. To functionalize the arrays for use they are first plasma cleaned to remove any organic matter from the surface of the array and add hydroxyl groups to the surface. Then they are dried and submerged in acetone to reduce the surface tension of the array to allow the micro-wells to fill. Arrays are then soaked in (3-Aminopropyl) triethoxysilane for silanization which will add amines to the hydroxyls. Arrays are then incubated in a mixture of dimethylformamide which acts as a solvent with pyridine and p-phenylene diisothiocyanate (PDITC). Because the PDITC solution is hydrophobic the next incubation with a chitosan solution prevents the chitosan from entering the microwells. Then only the surface of the array is coated. Following incubation in a vacuum, arrays are then quenched with an L-aspartic acid, NaCl, and Sodium Carbonate solution. This mixture coats the wells to prevent binding of mRNA's to the surface of the array. However, the chitosan coating allows for sealing and unsealing of the the polycarbonate membrane has a 10nm pore size which allows for the flow of buffers but not RNA transcript.

Single-cell Library Preparation: Single cell libraries were prepared following the published Seq-Well protocol [12]. In summary, 10,000 cells are loaded on to an array and sealed with a functionalized polycarbonate membrane. Cells are subjected to lysis and hybridization. STAMPS are removed from the array and put through reverse transcription overnight. The sample was then put through exonuclease digestion, second strand

synthesis, and whole transcriptome amplification. Samples were then tagmented using the Illumina NexteraXT kit.

Sequencing: Samples were sequenced on the Illumina Next-Seq 500 with 26bp Read1 and 50bp Read 2. Raw data was processed using Picard (Broad Institute, MIT)[44]. Cell and molecular barcodes were sorted and any sequences with a phred score of less than 10 are removed. Any potential extra adapter sequence is removed. PolyA sequences are trimmed based on any sequence with more than 6 sequential A sequences. The data set was then aligned to the GRCm38 (mm10) genome. After tagging the reads for digital gene expression analysis the sample was analyzed using Seurat [45]. First, any genes with an average expression less than 5 are removed from analysis. Any cells with less than 150 genes were filtered out, any cells with higher than 30 percent mitochondrial reads were filtered. The sample is then normalized using global-scaling normalization. Then the data goes through linear transformation to scale the dataset. Principal component analysis is then performed. The elbow method was used to determine significance of PC's and the first 15 PC's were used in analysis. Clusters are then determined with a resolution of 0.5 and visualized using t-SNE. Cluster identity was determined by known population markers. These include but are not limited to the following. RBC's (Hbb-bt, Hbb-bs, Hba-a1), B-cells (Immunoglobulin genes and Mzb1), Monocyte (Macrophages); (Lyz2, Cxcl2, Il1β), Enterocytes (Clca4, Cdh17, Hnf, reduced Tff3), Enteroendocrine (multiple protease genes, Reg4,), Goblet (Tff3), Fibroblasts (col3a1, col1a2, col1a1, col14a1, col5a1). Two populations were too small to distinguish a cell type and one epithelial population did not have enough distinct features to categorize.

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	Drop-Seq	10x Genomics;Chromium System	Seq-Well
Capture Efficiency	12.80%	50%	80%
Cost	~$0.26	~$0.60	~$0.13
Doublet Rate	0.36%-11%	~9%	1.8-11%
Genes/cell	~6,700	~4,500	~6,100
Transcripts/cell	~44,000	~27,000	~32,000

Table 3.1 Comparison of popular scRNA-seq platforms. Values for capture efficiency, doublet rate, genes/cell, and transcripts/cell are based off of data reported by individual platforms. Cost may vary based on sequencing costs and number of cells sequenced.

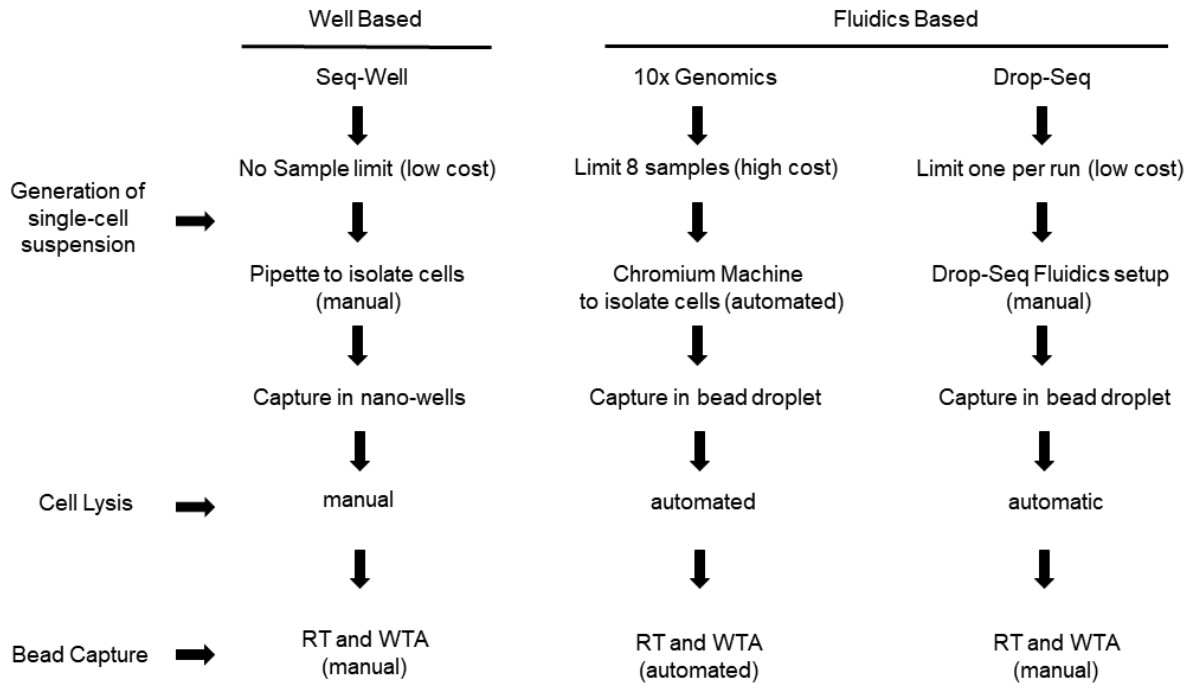

Figure 3.1 Workflow of single-cell sequencing platforms. Overview of automated and manual steps of single-cell platforms. RT; reverse transcription. WTA; whole transcriptome amplification.

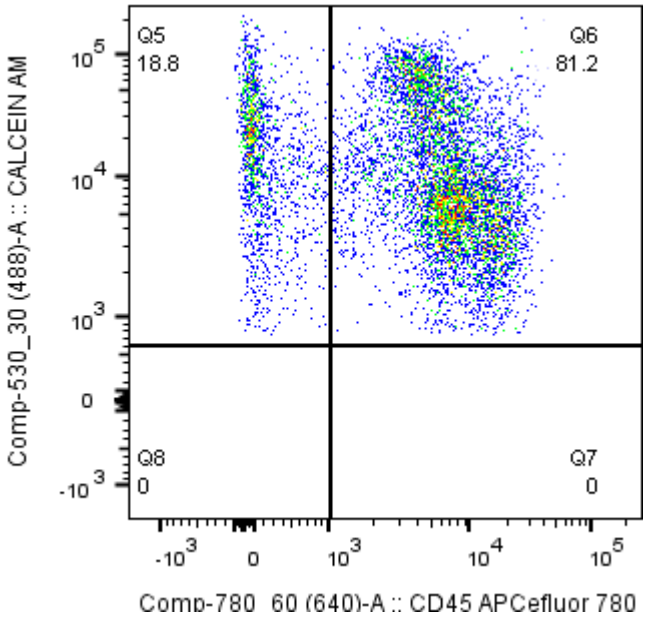

Figure 3.2 Validation of viable isolated CD45+ immune cells. Flow cytometry validation of Calcein+/CD45+ immune cells from colon of TripleMut mice.

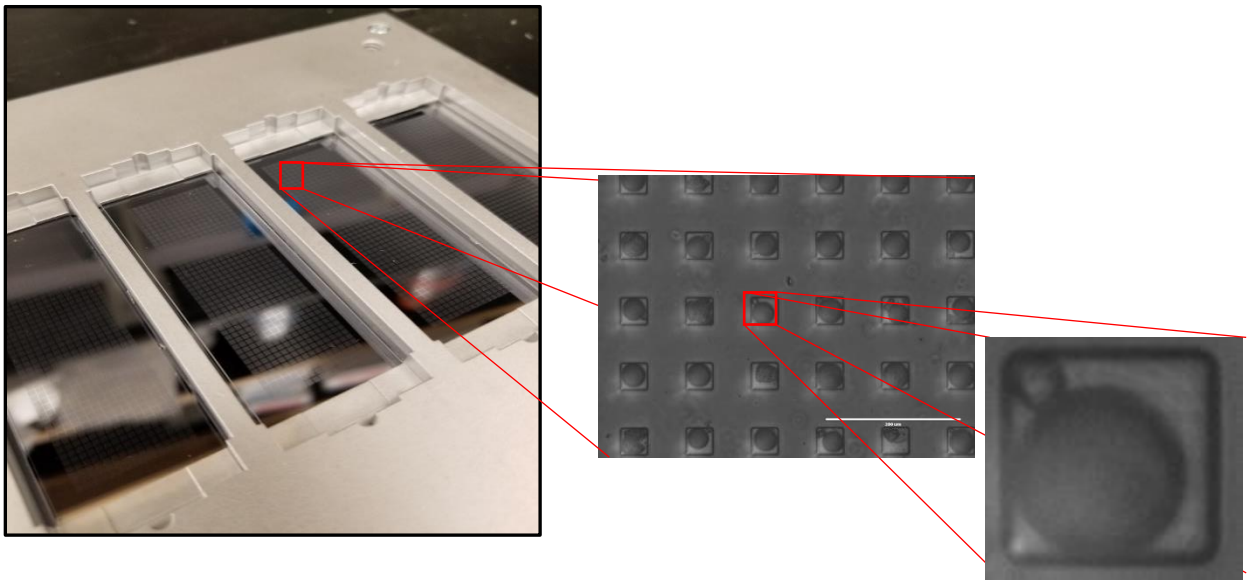

Figure 3.3 Functionalized and loaded Seq-Well array. Image of array mold and microscopic view of loaded seq-well array. Image of individual well containing larger barcoded bead and smaller cell.

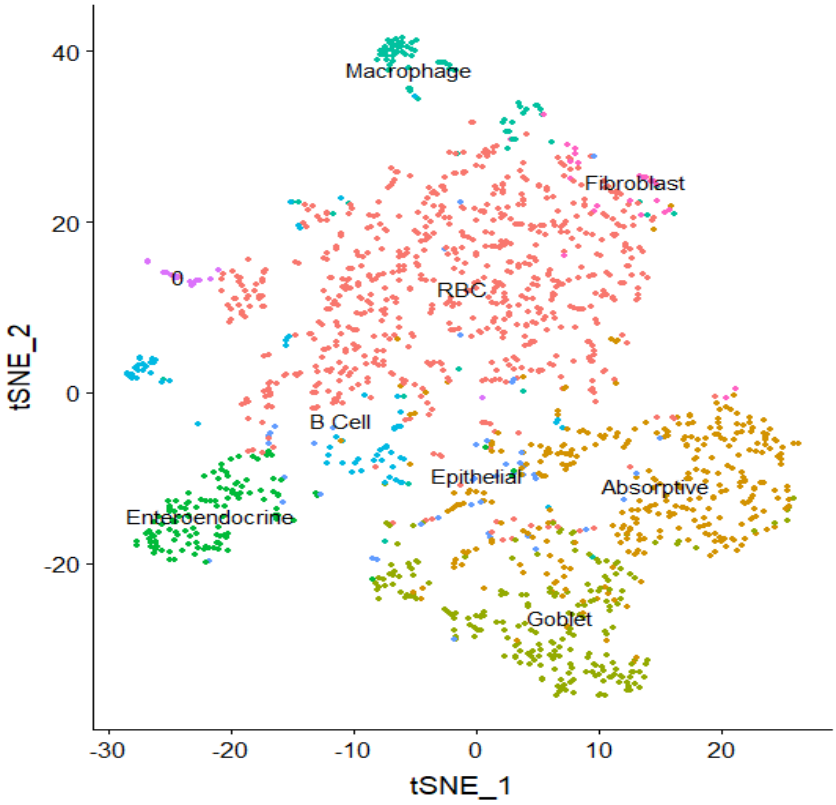

Figure 3.4 Identification of colon cell types with scRNA-seq. t-SNE plot of single cells from TripleMut mice after immune cell enrichment.

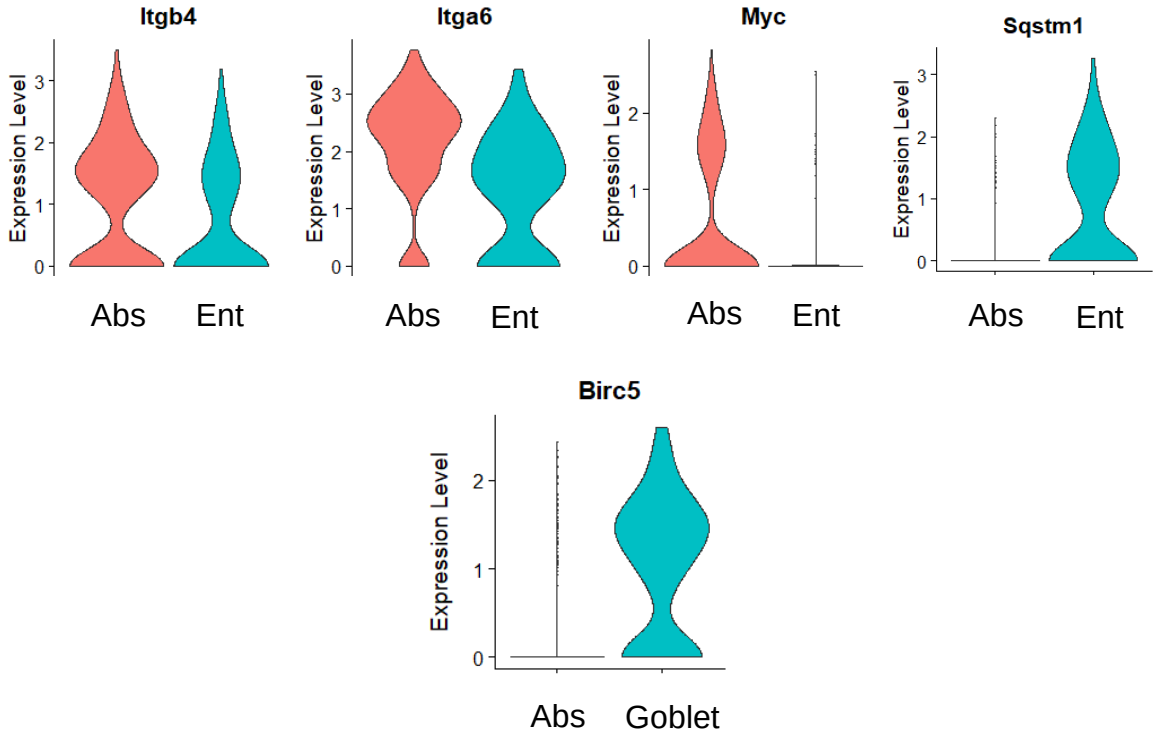

Figure 3.5 Enterocytes upregulate autophagy in CRC. Expression level comparison of significantly different autophagy associated genes between absorptive (Abs) and enteroendocrine (Ent) cells and Abs and Goblet cells.

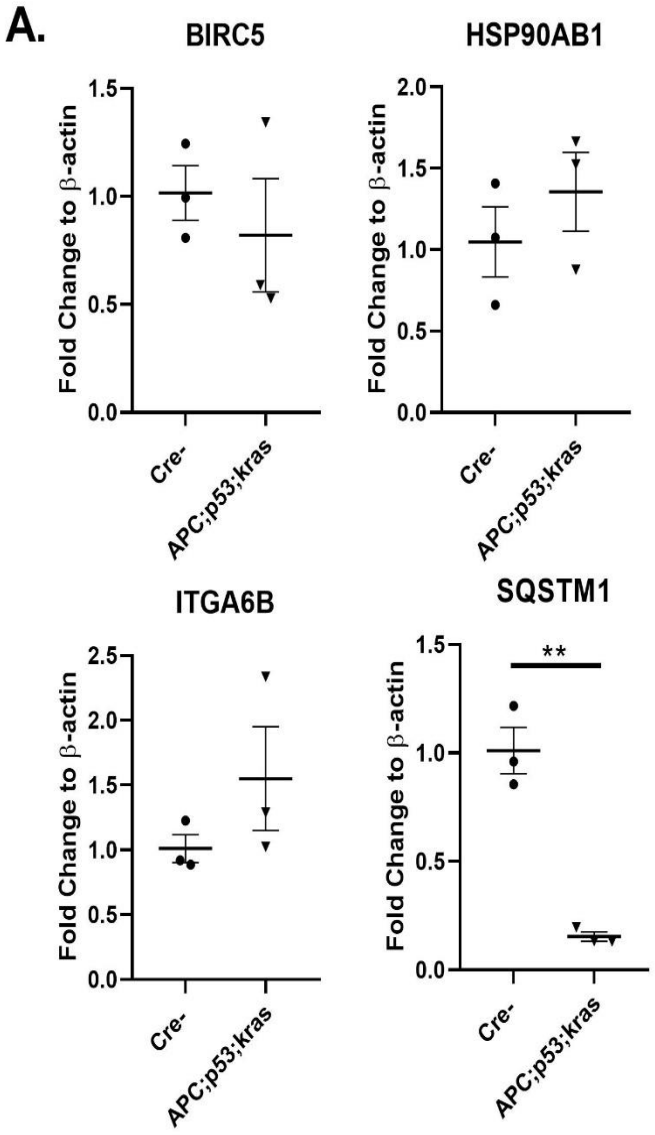

Figure 3.6. Autophagy differences are undetectable via bulk RNA analysis when compared to normal tissue. qPCR of autophagy genes from bulk colon tissue in TripleMut mice. ** p<0.01. Error bars represent SEM

Table 3.2 qPCR primers

Gene	Primer
BIRC5 Fw	GAACCCGATGACAACCCGAT
BIRC5 Rv	TGGCTCTCTGTCTGTCCAGT
HSP90AB1 Fw	ATGATCGGGCAGTTTGGTGT
HSP90AB1 Rv	CACCACTTCCTTGACCCTCC
ITGa6B Fw	ACCTCAATGCAGATGGGTGG
ITGA6B Rv	TAAACTGCACCCCCGACTTC
SQSTM1 Fw	TCTGGGGTAGTGGGTGTCAG
SQSTM1 Rv	AGAATGTGGGGGAGAGTGTG

Chapter 4

Conclusions

The tumor microenvironment is a highly complex and heterogeneous mix of cell types with pro- and anti-tumor signals. Tumors are comprised of tumor cells, tumor associated fibroblasts, immune cells, and extracellular signals from blood supply and oxygen availability. A major factor that determines tumor growth is the availability of extracellular and intracellular nutrients. In addition to cellular metabolic re-programming tumor cells utilize a number of mechanisms to maintain a nutrient pool for proliferation [1-4]. It is well known in cancer that autophagy can impact not only growth, but resistance to drugs, and ability to metastasize [5]. Unfortunately, conflicting literature suggests pro- and anti-tumor roles of autophagy in colon cancer [6-9].

This dissertation outlines a novel cell-autonomous role for autophagy in nutrient acquisition to promote tumor growth. I have shown that colon cancer cells rely on autophagy and specifically employ mitophagy for proliferation. In both sporadic and colitis associated cancer models, autophagy loss significantly reduces tumor growth. The reduction is not due to microenvironmental factors, rather an intrinsic reliance on autophagy. In the overall context of studying autophagy in tumor development, my work highlights the significance of understanding internal cellular cues for growth. I discuss these findings in the context of available literature to underscore the importance of

considering tumor stage and mutational load when studying the impact of autophagy on growth or response to therapy.

Cell-autonomous role of autophagy

Chapter 1 outlines the conflicting data in the field in which autophagy performs both tumor promoting and tumor suppressive roles. Signaling in the tumor environment provides non-autonomous cues for tumor progression. Non-cell autonomous cross-talk from modulating epithelial autophagy is known to impact the microbiome and immune system to modify tumor growth in CRC [10]. Moreover, in pancreatic cancer, cells signal to pancreatic stellate cells to induce autophagy to release alanine into the extracellular space that can be taken up by cancer cells for growth [4]. However, there is little evidence of the cell-autonomous roles of autophagy in colon cancer. I first assessed how loss of autophagy through *Atg5* deletion decreased tumor number, size, and proliferation in sporadic (*Cdx2-ER^{T2}Cre;Apc$^{fl/fl}$;Atg5$^{fl/fl}$)* and colitis (AOM/DSS) associated cancer. The sporadic and colitis models do not show changes in immune markers or immune cell infiltration. This is possibly due to housing differences and standardizing the microbiome. Interestingly, when autophagy was inhibited after tumor development in *Vil-ER^{T2};Atg5$^{fl/fl}$* mice, tumor burden was not significantly different with loss of *Atg5.* These findings suggest that autophagy may be important in early tumorigenesis but this observation should be studied in more depth (**Figure 4.1A&B**). Available literature identifies the importance of autophagy in preventing tumor development [11]. For example, autophagy may prevent DNA damage and tumor initiation [12]. Differences in the stage of tumor development may explain discrepancies between studies in CRC. Future work must place

an emphasis on understanding the impact of autophagy at different stages in tumor development.

I sought to uncover what cell-autonomous mechanisms inhibited tumor growth. Both monolayer and 3D cell culture methods showed a sensitivity to both genetic and pharmacological inhibition to autophagy suggesting a reliance on autophagy for survival. One study found that HCT116 and SW480 cells do not respond to autophagy inhibition similarly *in vivo.* Contrasting responses may suggest extracellular cues that impact autophagy. While this is a possibility, the limitation to this study is the use of the chorioallantoic membrane assay to assess growth which may impact cell growth due to species cross-reactivity [13]. Overall, cancer derived cell lines and patient-derived organoid models in this study showed a cell-autonomous dependency on autophagy, but the mechanism remained unknown.

Available literature on autophagy suggests that the process is utilized for nutrient acquisition under stress. However, little is known on exactly what nutrients are targeted or used by autophagy in colon cancer. To address this question, I screened cell growth in limiting nutrient growth conditions and found that loss of heat labile proteins in combination with autophagy inhibition reduced growth. To assess what nutrients were dependent on autophagy, I performed metabolomic analysis. The most strongly impacted pathway was a decrease in metabolites associated with the TCA cycle. Further work is necessary to clearly identify the autophagic dependent nutrients essential to promote cell proliferation either individually or in combination. Moreover, analysis of the top hits showed a significant decrease in cysteine and methionine metabolism under $Serum^{HI}$. Cysteine is a well-known amino acid that can be oxidized by reactive oxygen species

(ROS) [14]. A potential cause of the observed growth inhibition could be due to increased ROS due to a reduction in cysteine. I did investigate whether supplementation with N-acetyl-cysteine, a precursor to cysteine, could rescue growth inhibition. Treating cells with N-acetyl-cysteine did not rescue cell growth (data not shown). Similar to cysteine, methionine can affect redox reactions and is also known to impact nucleotide metabolism in cancer [15]. Because of the high nutrient demand in cancer cells, the requirement of several metabolites may be necessary to rescue growth.

An important limitation to consider when assessing these studies is that the tumor microenvironment and the nutrients available are significantly different than that in cell culture conditions. For example, cancer cells consume different amounts of glutamine based on their *in vitro* versus *in vivo* culture [16]. In lung cancer, environmental cysteine impacts cells reliance on glutamine and this difference is observed in culture versus in the tumor [16]. New tools to eliminate artifacts from cell culture media such as more physiological relevant media are being generated to allow for more accurate assessment of colon cancer response to available nutrients [17].

It is important to confirm the cell-autonomous role of autophagy in nutrient acquisition in *in vivo* models. Dietary nutrients contribute to the development of colon cancer and have been used as supplement to therapeutic treatments. Diet and nutrient interaction in colon tumors is an active area of research. Intermittent fasting and caloric restriction can impact tumor growth and progression [18, 19]. Iron is known to increase risk of colon cancer [20]. A high fat diet increases the risk of colon cancer development [21-23]. Additionally, increase in dietary glucose and fructose also increase risk for colon cancer [24, 25]. It is possible that an overabundance of nutrients allows colon cancer cells

to bypass the need for autophagy. Under stress and changes in the tumor microenvironment, cells upregulate autophagy to acquire nutrients.

Since nutrient restriction is found to impact tumor growth, I assessed if nutrient restriction, in combination with autophagy loss, could synergistically reduce tumor growth. To investigate the role of nutrient limitation in combination with autophagy loss I used xenograft models where mice were placed on caloric restriction for a week. The mice were injected with MC38 mouse adenocarcinoma cell line, 10 days later, injected once daily with chloroquine. Mice on 25% caloric restriction with chloroquine did not show a significant reduction in tumor growth (**Figure 4.2A&B**). In many of the previous models investigating caloric restriction and tumor growth, mice were calorie restricted significantly longer than the present study. Many of the tumors in the present study developed ulcerations leading to the shortened timeline. Tumor size did decrease in the caloric restriction/autophagy cohort so it is possible that with longer treatment I may observe a more robust phenotype. In a second model, the TripleMut mice showed no difference after two weeks of chloroquine treatment (**Figure 4.3A-D**). Dysplastic foci were not changed after chloroquine treatment. However, due to the highly proliferative nature at two weeks following induction in the TripleMut mice it may be more advantageous to perform these experiments following a low dose of tamoxifen. Similarly, low dose tamoxifen should be done in combination with longer treatment of chloroquine. However, in order to observe autophagy loss on developed tumors, treatment should be started at a timepoint after tumors have developed.

I have shown a reliance on autophagy and susceptibility to growth inhibition with loss of nutrients in $Serum^{HI}$. It is still unclear how the cell senses this nutrient limitation and

signals for autophagy, or mitophagy to be upregulated. A main contributor to nutrient sensing in the cell is molecular target of rapamycin (mTOR) [26]. Nutrient signals from glucose and amino acids can activate or inhibit mTOR [27]. Autophagy and mTOR have a dynamic relationship in which the activity of one impacts the other [28]. The nutrients sensed through mTOR may regulate autophagy activation or manipulation of the mechanisms between these two pathways may alter normal cell homeostasis [29]. Investigation of the cross talk between these pathways may shed light on the mechanisms activating autophagy. mTOR regulation should be assessed under different nutrient stresses such as $Serum^{HI}$. When amino acids are abundant, mTOR is activated on the lysosomal surface. Loss of available amino acids will inhibit its activation. The acquisition of mitochondrial metabolites through mitophagy does impact cell growth as discussed in Chapter 2. However, there was an overall increase in peptides present in the lysosome under nutrient stress. It is possible that reduced growth is partially attributed to overall reduced nutrients and inhibition of mTOR. Another caveat to consider with chloroquine treatment is that the mTOR activation on the lysosomal surface may be hindered by the pH change induced by chloroquine[30].

This work primarily focused on the role of autophagy and cell growth. However, autophagy is known to modulate metastasis and response to chemotherapeutics. Common chemotherapeutics may modulate the activity of autophagy and whether or not it enhances or blocks tumor growth. Combination therapy with autophagy inhibition and chemotherapy may prove efficacious for some patients. Similarly, dietary modulation in combination with chemotherapeutics may positively impact autophagy to suppress tumor growth.

Mitophagy in colon cancer

Mitophagy has similar conflicting data where it is both pro- and anti-tumorigenic [31, 32]. Activation of mitophagy may also be dependent on location within a tumor, either the deep tumor or invasive front [33]. The present study defines mitophagy as a pro-tumor function in epithelial cells to support proliferation. When PRKN or PINK1 are knocked down in colon cancer cell lines, a profound loss of cell growth is observed even in nutrient rich conditions. The data suggests that colon cancer cells have adapted to employ mitophagy in nutrient rich environments. There are different mechanisms outside of the PRKN/PINK1 mediated pathway that induce mitophagy and it is possible that colon cancer cells employ alternate mechanisms [34, 35]. Conversely to the data in this thesis, one study identified that activation of mitophagy via treatment with Mito-Met_{10} (a complex I inhibitor) can abrogate cell growth [36]. Mito-Met_{10} can also act on AMPK and cell cycle regulators which may explain the decrease in growth compared to mitophagy inhibition alone.

A limitation to this dissertation is the models used to explore mitophagy in colon cancer. Due to the differences between *in vitro* and *in vivo* models the utilization of a tumor model will provide tools to better study this mechanism in tumor development. I have crossed a *Prkn*$^{fl/fl}$ mouse with the *Cdx2-ER*^{T2}*Cre; Apc*$^{fl/fl}$ model and a *Clec16a*$^{fl/fl}$ mouse with the *Cdx2-ER*^{T2}*Cre; Apc*$^{fl/fl}$. Clec16a modulates NRDP1-PRKN regulation. The loss of Clec16a enhances expression of PRKN to increase mitophagy [37]. With these mice, sporadic tumor development can be studied with the presence or absence of mitophagy. Similarly, the *Prkn*$^{fl/fl}$ and *Clec16a*$^{fl/fl}$ mice can be placed on the AOM/DSS protocol to observe loss or activation of mitophagy prior to tumor development or after

tumors have been developed. These models allow for the assessment of tumor number, size, and burden. While I did not observe immune cell changes in the autophagy models used in this dissertation, immune cell infiltration should be assessed. Mitophagy is found to impact innate immunity in the cell underscoring the importance of investigating different facets of mitophagy in the cell [38, 39]. However, since this model utilizes a colon specific Cre-recombinase, any changes observed in immune cells will be due to changes in cellular cues from epithelial cells. One limitation to these models is that they impact the PINK1/PRKN mediated form of mitophagy. When alternate mechanisms of mitophagy in colon cancer are identified, mouse models to study these pathways in tumor development may be useful.

While the *Atg5* models I used in this study uncovered a cell-autonomous role for autophagy, complete loss of macro-autophagy through *Atg5* depletion may impact tumor growth differently than that of mitophagy alone. It is possible that a combination of nutrients taken up in the lysosome in addition to mitophagy are contributing to the growth inhibition. Inducible shRNA for PRKN or PINK1 can be designed to target mouse PRKN/PINK1 and stably transfected in to mouse CT26 or MC38 cells. The same cell lines can simultaneously be transfected with TMEM192-3xHA. Using these cell lines, xenografts of TMEM192-3xHA/PRKN shRNA expressing cells can be induced with or without doxycycline and lysosomes can be isolated. Proteomic or metabolomic analysis will allow for exploration of changes in lysosomal content following mitophagy inhibition. This experiment will also provide further insight on tumor growth with or without mitophagy.

This dissertation uncovered the role of mitophagy in CRC but other selective forms of autophagy may be utilized in the cell as discussed in Chapter 1. It is possible that mitophagy is primarily employed by specific cell types within the colon epithelium. In colon cancer stem cells, resistance to doxyrubicin was attributed to an influx in mitophagy. Mitophagy was increased in cancer stem cells compared to the parental cells and led to their increased resistance [40]. Mitophagy induction was through a BNIP3 directed fashion further underscoring the importance of PRKN-independent mitophagy. In colon cancer stem cells, mitochondrial oxidative phosphorylation is increased to maintain their proliferative function [41]. Mitophagy may help to maintain the balance of oxidative phosphorylation and reduced ROS in these cells. While mitophagy has not been investigated in stem cells, future work to understand if basal levels of mitophagy are used in this cell population for maintenance may highlight a new clinical target.

When the cell utilizes autophagy to break down organelles and proteins, the products are recycled for cellular use. This dissertation has identified mitochondria as one of the degradative substrates to target. However, what these metabolites are used for following release in to the cell is not known. Mitochondrial metabolites are known to contribute to cellular functions outside of the TCA cycle [42]. It is possible to utilize isotope tracing to assess differences in mitochondrial metabolites under nutrient stress [43]. However, this will not provide information on where the metabolites are being used in the cell, rather what metabolites are being generated.

One potential impact of inhibiting macro-autophagy or selective mitophagy is the accumulation of dysfunctional mitochondria. The cytotoxicity of these mitochondria may reduce cell growth and lead to cell death. Under these conditions it would be important to

measure mitochondrial respiration using assays such as Sea Horse developed by Agilent. Mitochondria function can also be assessed using immunofluorescence and common mitochondrial markers can be compared to measure dysfunctional mitochondria. Further investigation on the role of mitophagy is needed in CRC.

Single-cell modulations of autophagy

The epithelial cell specificity discussed in this dissertation outlines the importance of elucidating the mechanistic role of pathways in the hetero-cellular tumor environment. While I have identified a cell-autonomous role of autophagy in colon cancer growth, autophagy modulation in different colon epithelial cells has not been investigated.

In a mouse model with loss of *Apc, p53,* and a mutant $Kras^{G12D}$ I isolated colon tissue and performed single-cell sequencing using the Seq-well platform. I identified multiple colonic epithelial cell types including secretory and absorptive cells in addition to different immune cell types. Within the tumor tissue, increased autophagic gene expression was higher in absorptive compared to secretory cells. No changes in immune cell-autonomous autophagy was observed. One possibility for the cell type specific autophagy expression is that absorptive cells are essential in nutrient uptake from the extracellular environment and employ autophagy for breakdown and use. As previously discussed, Paneth cell autophagy impacts the intestine in inflammatory bowel disease [44]. Moreover, histological analysis determined that goblet cells are found to be enlarged following autophagy inhibition in the colon [45]. Single-cell transcriptomic analysis is unable to inform researchers about morphological changes which is why this observation may not have been found in this study. Single-cell sequencing may be used in

combination with new advanced techniques that provide in-depth spatial information [46]. Importantly, many of the autophagic changes with single-cell resolution were not detectable in bulk RNAseq analysis. When comparing the autophagy signature in whole tissue RNA sample, many of the markers were not different when compared to controls. This is in part due to the presence of immune cells and the heterogeneous mixture of epithelial cells.

Future studies should aim to understand how autophagy is modulated temporally through tumor development. The work in this dissertation and previous literature highlight the fluctuation of autophagy in the tumor. While mouse models of colon cancer are an invaluable tool for cancer research, often times they do not recapitulate the mutational landscape in patients [47]. Common mutations in colon cancer include *APC, p53,* and *KRAS* among others, but each patient has a unique mutational load. For patients, treatment strategies will vary based on mutations, stage, and location of tumor [48]. Chapter 1 briefly outlines autophagy mutations present in colon cancer as a prognostic tool [49]. Autophagy specific mutations in combination with other common mutations in CRC may modulate the impact of how autophagy modulates tumor growth. One example of this complexity is that p53 can modulate autophagy by regulating LC3 under starvation [50]. No immune differences were found in this study but the known cross-talk between immune and tumor epithelial cells emphasizes the need to understand how autophagy impacts the tumor as a whole. The limitations from current mouse models, and complexity of mutational landscape may be addressed using single-cell sequencing from patient derived samples. Seq-well is a cost-effective platform that enables processing of multiple samples simultaneously and may be a beneficial tool to explore these questions.

Throughout this dissertation I have primarily focused on autophagy at the primary tumor sight. When considering tumor stage, it is important to evaluate the metastatic roles of this mechanism. Similar to the primary tumor site, autophagy is found to perform pro and anti-metastatic roles [51]. Little work has been done specifically in colon cancer to address this question. Single-cell sequencing can be applied to metastatic nodes in colon cancer models or from those in patients. Single-cell sequencing may uncover specific cell types that metastasize and how autophagy is altered in metastatic versus primary tissue. Metastatic models are available to assess these questions *in vivo* [52]. Understanding autophagy in metastatic sites will help guide researchers and clinicians in developing therapies and enhancing individualized patient care for those with advance stages of disease.

Therapeutic approaches

Over the past few decades, interest in targeting autophagy for cancer treatment has increased. Therapies directed at these mechanisms may prove effective in reducing tumor burden. Hydroxy-chloroquine (HCQ) is currently the only clinically approved autophagy inhibitor. Unfortunately, we did not observe significant decrease in tumor growth using chloroquine *in vivo.* This data is consistent with others due to limitations in the pharmacokinetics and achieving the proper circulating levels to inhibit autophagy [53, 54]. The development of more specifically targeted drugs may allow for better treatment that can be effective at low doses. I demonstrated that the use of SBI-0206965, a ULK-1 inhibitor is able to block cell growth. This compound has the potential to be used as a more targeted approach of autophagy inhibition following further safety and efficacy studies [55]. I utilized a dominant negative ATG4B to inhibit growth, the development of

inhibitors NSC185058 and SC377071 which target ATG4B and suppressed osteosarcoma growth may also prove beneficial [56]. The functional role of autophagy in different cell types is a confounding factor in using therapies targeted toward this mechanism. If tools can be utilized to target autophagy in epithelial cells, and not in immune cells, it may provide a more efficacious therapy. A promising approach is the utilization of nanoparticles. Using nanoparticles, siRNA can be selectively delivered to endothelial cells [57]. Nanoparticles can also deliver genes to cells based on cell-specific promoters [58]. Drugs can target the tumor via coating nanoparticles with cancer cell membrane [59]. I have shown that loss of autophagy in epithelial cells impacts growth selectively in tumor cells and does not impact the growth of normal colon cells. Autophagy can be specifically targeted in epithelial cells while preventing impact on immune cells with this approach as would occur with pharmacological inhibitors.

This thesis has identified targeting of mitophagy as a potential approach for CRC treatment. Pharmacological methods can be used to inhibit mitophagy such as mitochondrial division inhibitor (Mdivi1) which inhibits Drp1 activity [60, 61]. There is limited bioavailability of potential mitophagy inhibitors. The utilization of siRNA to target modulators is a potential approach. While this dissertation primarily focuses on PINK1/PRKN mediated mitophagy, other mechanisms of mitophagy may be important to acquiring metabolites and targeting these pathways for therapy may be beneficial. If tumor cells are adapting to alternate mechanisms the treatments will need to be assessed on an individual patient basis.

Final Thoughts

This dissertation uncovers a novel role for autophagy in colon cancer growth. This work further defines the importance of understanding cell type specific modulations of autophagy. While the field of autophagy research in colon cancer is relatively new, the potential ability to target this mechanism is efficacious. This data serves as a framework for targeting mitophagy as a treatment for colon cancer. Understanding the nutrient sensing and mechanisms that activate autophagy in the tumor will inform ways that this pathway can be targeted for therapy. Similarly, I have shown the cell-autonomous role of autophagy for cell growth but little work has been done in colon cancer on the cell-autonomous effects on metastasis and response to therapy. Future work may determine how autophagy modulation changes during different stages of tumor development and how this information can improve individualized patient care.

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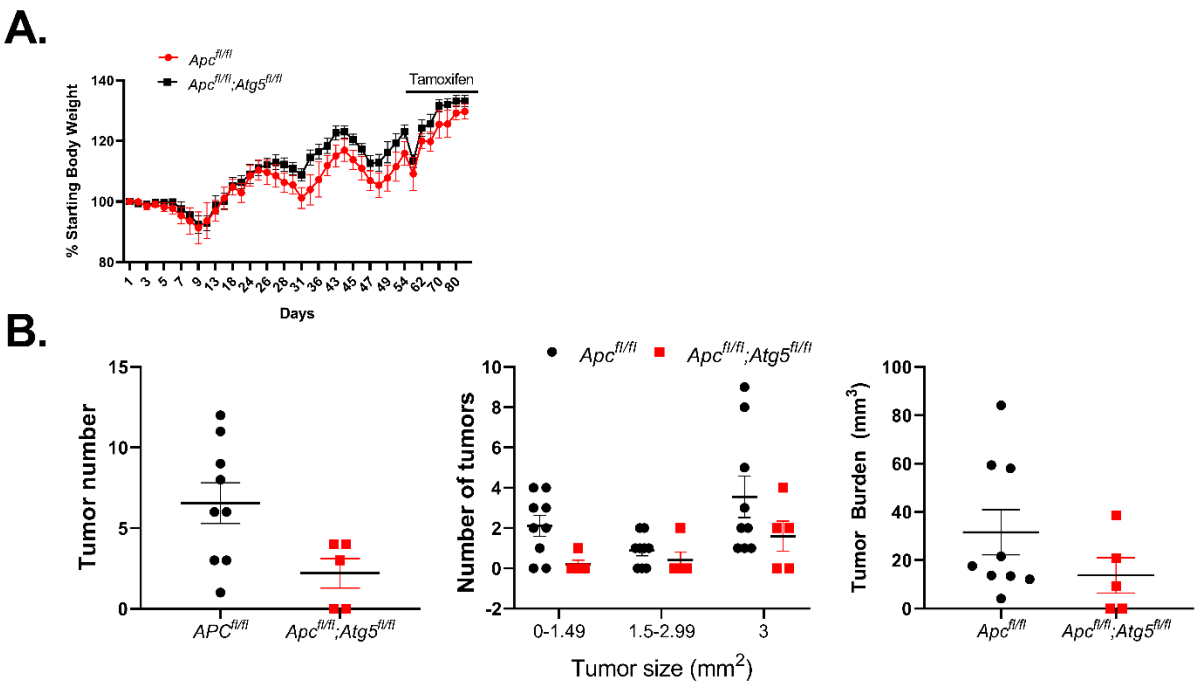

Figure 4.1 Loss of autophagy in late tumor development does not significantly reduce tumor growth in colitis associated cancer. A. Weights of mice on AOM/DSS. **B.** Tumor number, tumor size, and tumor burden. Error bars represent SEM.

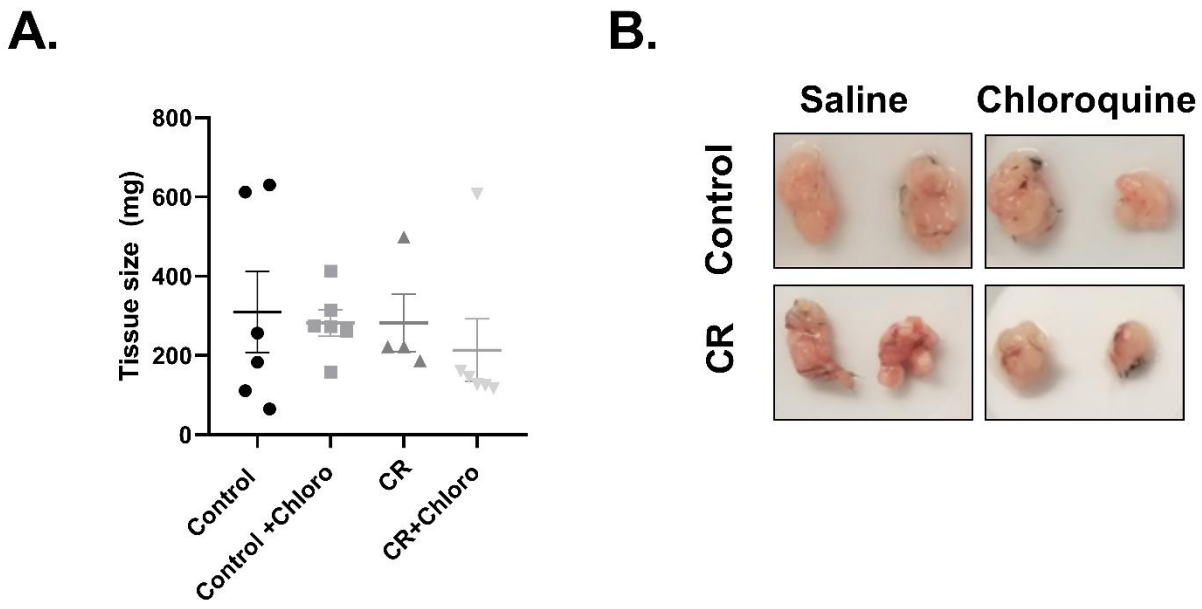

Figure 4.2 Caloric restriction and pharmacological inhibition of autophagy does not significantly reduce growth in xenograft models. A. Quantification of tumor size. **B.** Representative images of tumors from individual mice. Error bars represent SEM.

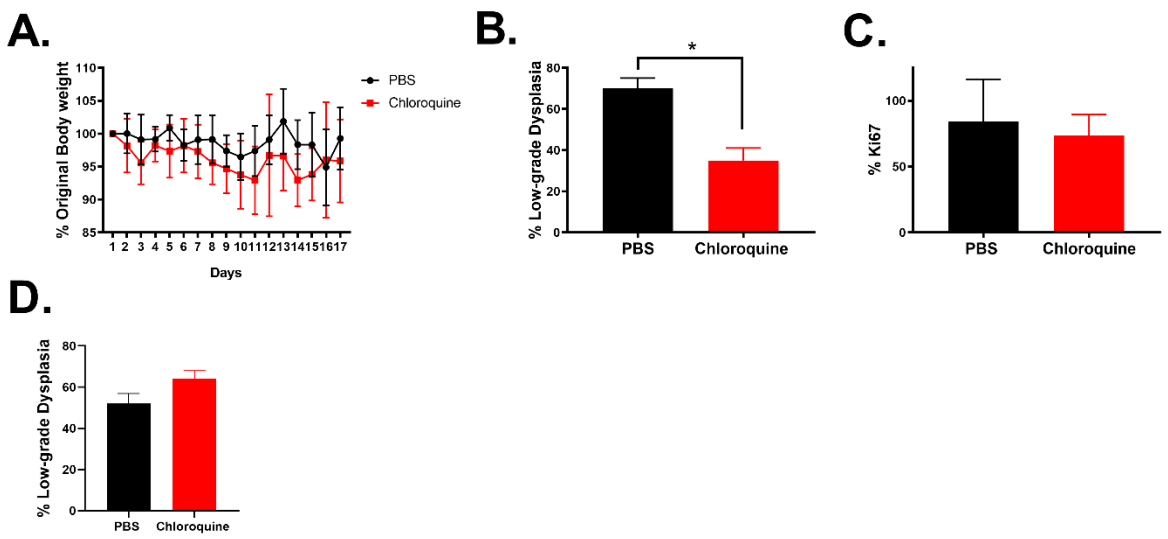

Figure 4.3 Inhibition of autophagy in spontaneous tumor model does not reduce low-grade dysplasia at tumor initiation. A. Weights of mice treated with chloroquine. **B.** Dysplasia scoring by blinded pathologist from H&E sections. **C.** Percent Ki67 positive staining. **D.** Dysplasia scoring of second cohort of mice treated with chloroquine. * p <0.05. Error bars represent SEM.