Starving Cancer from the Inside and Outside: Nutrient Stress in Combination with Autophagy Inhibition to Kill Pancreatic Ductal Adenocarcinoma (PDAC)

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Abstract:

Pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest cancers worldwide, with a 5-year survival rate of less than 5 percent. Given the current lack of effective therapeutic options, novel strategies for treatment of this disease are urgently required. Most PDAC tumors acquire mutations that result in constitutive activation of the GTPase KRAS, which promotes mitogen-activated protein kinase (MAPK) signaling and tumor development. Notably, KRAS-driven PDACs often exhibit increased dependence on autophagy, the process by which cells degrade internal components to mobilize energy stores. When faced with cellular stress, such as nutrient or growth factor deprivation, tumor growth may initially slow in response to decreasing supplies of growth factors and cytokines. Under these conditions, the induction of autophagy can enable continued tumor growth even in the presence of nutrient stress, and PDAC cells have been demonstrated to upregulate autophagy to maintain pro-tumorigenic growth. Therefore, we posited that nutrient restriction in combination with autophagy inhibition would synergistically disrupt aberrant metabolic pathways and more effectively stunt PDAC tumor progression. To determine the impact of transient autophagy inhibition, the aminoquinoline chloroquine (CQ, the only FDA-approved autophagy inhibitor) was selected. The combination of CQ and nutrient stress elicited a compounding effect on cellular proliferation in both murine and human-derived PDAC cells *in vitro*, demonstrating the potential efficacy of CQ and nutrient stress as an adjuvant therapy for PDAC. Next, the mechanistic impact of permanent autophagy inhibition was explored, in order to identify secondary metabolic alterations arising in response to autophagy ablation. Using CRISPR/Cas9 technology, a key gene in autophagic induction, autophagy related 5 *(Atg5)* was deleted in a murine-derived KRAS-mutant PDAC cell line (Panc02) rendering them autophagy-deficient ($Atg5^{-/-}$) PDAC cell line. To understand the effect of systemic nutrient deprivation and

autophagy deficiency, these Atg5 wildtype and knockout cells were used in an *in vivo* model in which animals were prescribed a control or calorie restricted diet. Upon intrapancreatic injection in a syngeneic C57BL/6 model, the complete loss of autophagy in $Atg^{-/-}$ cells resulted in reduced tumor growth relative to control ($Atg5^{+/+}$) cells. However, no differences in growth were observed *in vitro* under standard growth conditions or in combination with nutrient stress, indicating the cells with permanent autophagy deficiency have reprogrammed to grow in an autophagy-independent manner. Thus, our findings reveal a potential synergism between autophagy inhibition and nutrient stress, and also caution against suppressing autophagy completely and/or permanently due to the potential for pro-survival cellular adaptation to occur.

Introduction:

Pancreatic Cancer Prevalence and Mortality

Pancreatic cancer is one of the deadliest malignancies worldwide, with a 5-year survival rate less than 5 percent (Siegel, Miller et al. 2018). In the United States, pancreatic cancer is currently the 4th most fatal cancer for both men and women, and is expected to become the 2nd leading cause of cancer-related deaths within the next decade (Rahib, Smith et al. 2014, Siegel, Miller et al. 2017). Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer (Kleeff, Korc et al. 2016). While little is known about the etiology of pancreatic cancer, novel strategies for treatment of PDAC are urgently needed. The vast majority of PDAC tumors have an oncogenic activation of the GTPase KRAS, which promotes mitogen-activated protein kinase (MAPK) signaling and tumor development (Min-Jung Kim 2011). Notably, autophagy, an intracellular catabolic process that disassembles cellular components to provide energy and nutrients to cells, is often heightened in KRAS-driven PDAC (Min-Jung Kim 2011). Thus, the process of autophagy has emerged as a potential therapeutic target for PDAC.

Autophagy

Autophagy is a highly conserved process that degrades and recycles cellular organelles and other cytoplasmic components (Kuma and Mizushima 2010, Ohsumi 2014). While there are multiple autophagic pathways, including microautophagy and chaperone-mediated autophagy, in this thesis the term autophagy refers to a process also known as macroautophagy, wherein an isolation membrane, or phagophore, sequesters protein aggregates or organelles into autophagosomes for transport to the lysosomes for degradation (Mizushima, Yamamoto et al. 2004, Kuma and Mizushima 2010, Mizushima and Levine 2010). Thus autophagy is a key homeostatic process, removing redundant or damaged organelles, thereby generating metabolites

used to provide energy or create new macromolecules (Boya, Reggiori et al. 2013). Autophagy is predominately regulated via the phosphoinositol 3-kinase/mammalian target of rapamycin complex I (PI3K/ mTORC1) pathway, a major orchestrator of cellular nutrient and energy balance (Jung, Ro et al. 2010, Saxton and Sabatini 2017). When energy stores are high, mTORC1 negatively regulates autophagy; however, under conditions of nutrient stress or starvation mTORC1 activity declines, leading to the induction of autophagy above basal levels (Levine and Kroemer 2008, Efeyan and Sabatini 2010, Jung, Ro et al. 2010).

Autophagy and Cancer

Autophagy has a complex role in cancer, as its effects appear to vary during different stages of cancer progression (White 2015). In the context of carcinogenesis, autophagy is thought to exert protective effects, eliminating misfolded proteins and damaged cellular components. Defects in autophagy can result in metabolic disruption, increased misfolded protein burden, the generation of reactive oxygen species (ROS), and subsequent oxidative stress (Komatsu, Waguri et al. 2007, Mathew, Karp et al. 2009, Mizushima and Komatsu 2011). In untransformed cells, autophagy thereby serves a central role in mitigating stress and preserving genomic stability (Qu, Yu et al. 2003).

Conversely, in established tumors, autophagy may act as an essential adaptive response to overcome cellular stressors and promote tumor progression (White 2015), thus revealing autophagy as a "double edged-sword" in cancer (Huo, Cai et al. 2013). Indeed, autophagy is instrumental in generating metabolic fuels for tumors limited by dysfunctional vascularization, and hypoxic or nutrient deficient microenvironments, and thus plays a role in the progression of cancer towards metastatic disease. (Dewhirst, Cao et al. 2008, Zhang, Bosch-Marce et al. 2008, White 2015).

Metastatic disease is the primary cause of cancer related deaths (Weigelt, Peterse et al. 2005, Mehlen and Puisieux 2006, 2018). Importantly, the majority of PDAC cases are diagnosed in late stages of the disease, having spread beyond the confines of the pancreas (Siegel, Miller et al. 2018). Therefore, an improved understanding of autophagy's role in the biology of metastasis may offer novel therapeutic targets for PDAC (Thiery, Acloque et al. 2009). The epithelial-to-mesenchymal transition (EMT) is a de-differentiation process whereby epithelial cells lose their polarity and assume the more motile, invasive phenotype of mesenchymal cells (Lock and Debnath 2008, Guadamillas, Cerezo et al. 2011). In carcinoma cells, EMT elevates cancer cell presence in systemic circulation, thus increasing the likelihood of secondary tumor formation (Thiery, Acloque et al. 2009).

Autophagy Inhibition and Therapy

The "double-edged sword" of autophagy in cancer biology complicates the implications of targeting autophagy in cancer cells. Early studies investigating the mechanism of autophagy utilized genetic silencing of key autophagy genes such as *Atg5*, *Atg7*, *Atg12,* and *Becn1*, effectively disrupting the autophagy process to provide more insight into the molecular role of each autophagy related gene (Pattison, Osinska et al. 2011, Cufi, Vazquez-Martin et al. 2012, Kim, Jeong et al. 2012, Huo, Cai et al. 2013). Despite gaps in the current understanding of autophagy's complete role in cancer, these studies wherein autophagy is permanently disrupted in cancer cells support that inhibition of autophagy impairs tumor formation (White 2015, Lashinger, O'Flanagan et al. 2016).

Transient pharmacological inhibition of autophagy has been investigated for potential use as adjuvant therapy (Solomon and Lee 2009, Amaravadi, Lippincott-Schwartz et al. 2011, Long, Yang et al. 2013, Rainsford, Parke et al. 2015). Many of these studies employ chloroquine (CQ),

a known pharmacologic inhibitor of autophagy. CQ inhibits autophagy by preventing endosomal acidification, resulting in inhibition of lysosomal enzymes that require an acidic pH, and thus disrupting the maturation of the autophagolysosome (Solomon and Lee 2009, Amaravadi, Lippincott-Schwartz et al. 2011, Long, Yang et al. 2013, Rainsford, Parke et al. 2015). A Phase II pharmacodynamic study in patients with metastatic PDAC showed no significant effect in progression-free survival in response to a CQ derivative (Wolpin, Rubinson et al. 2014). However, in preclinical models of KRAS-driven pancreatic cancers, CQ has been shown to moderately reduce cell growth, tumorigenicity, and oxidative phosphorylation (Yang, Wang et al. 2011).

For the studies herein, we hypothesized that inhibition of autophagy under conditions in which autophagy dependence is increased – i.e., nutrient deprivation – would synergistically stunt PDAC tumor progression. Calorie restriction (CR) is a dietary manipulation which decreases caloric intake without incurring malnutrition, and also induces autophagy (Mitchell, Tang et al. 2015). CR has been shown to effectively reduce tumor burden in various types of cancer (Brandhorst, Wei et al. 2013, Champ, Baserga et al. 2013, Di Biase, Lee et al. 2016, O'Flanagan, Smith et al. 2017, Rossi, Dunlap et al. 2017). While CQ has worked to decrease tumor burden in preclinical models, treatment with transient autophagy inhibition may incur resistance to therapies that inhibit autophagy. We hypothesize that cells may upregulate compensatory pathways to sustain cell proliferation under conditions of chronic autophagy inhibition. Understanding the differences between transient, chronic, and permanent autophagy inhibition may allow for more effective treatment. This body of work analyzes pharmacological, transient and chronic inhibition compared to permanent, genetic autophagy deficiency in combination with nutrient stress in *in vitro* and *in vivo* models.

Methods:

Cell Culture

All cell culture experiments used Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), and 5mg/mL penicillin/streptomycin unless otherwise noted.

Western Immunoblots

Cells were lysed with radioimmunoprecipitation (RIPA) buffer supplemented with protease inhibiter (Roche) and Halt Phosphatase inhibitor (Thermo Scientific). Protein concentration was determined via Bradford Assay using a Bradford protein quantification reagent (Biorad). Forty micrograms of protein were loaded into a 4–15% stain-free gel (Biorad) and transferred onto a PVDF membrane using a Transblot Turbo transfer unit (Biorad). Membranes were blocked with 5% bovine serum albumin (BSA) in Tris Buffered Saline containing 0.1% Tween (TBST) for 1 hour. Membranes were then incubated with a primary antibody [LC3B I&II (Cell Signaling Technology, #3868S, Lot 13) (1:1000); β-Actin (Cell Signaling Technology, #4970L, Lot 14) (1:1000); Atg5 (Cell Signaling Technology, #12994S, Lot 3) (1:1000); PCNA (Cell Signaling Technology, #2586S) (1:1000); E-cadherin (BD Transduction Laboratories, #610181, Lot 4337639); N-cadherin (BD Transduction Laboratories, #4330954, Lot 4330954); Snail (Abcam, ab180714, Lot GR206562-1)] overnight at 4°C, followed by HRP-conjugated secondary antibodies raised against rabbit or mouse IgG (Sigma Aldrich). Proteins were visualized using a Chemi Doc MP system (Biorad) and blots were quantified using ImageJ software.

Mechanistic (Pharmacologic) Inhibition of Autophagy

CQ (Chloroquine Diphosphate Salt, Sigma #101352441) was dissolved in sterile, deionized H_2O to make a 10 mM stock that was aliquoted (100 μL) and frozen at -20°C. Wildtype mouse-derived Panc02 K-Ras driven pancreatic and wildtype human derived MIA PaCa-2 pancreatic cancer cell lines were treated with or without a therapeutic dose of 10 μM CQ for 48 hours for all experiments (Lashinger, O'Flanagan et al. 2016). Western Immunoblots were used to determine the extent to which autophagy was successfully inhibited.

Cell Proliferation

An MTT assay was used to measure cell proliferation over a 48-hour period. Panc02 and MIA PaCa-2 cells were seeded in a 96-well plate (5×10^3 cells/well). After 48 hours with or without CQ treatment, cells were stained with a solution containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.5 mg/mL) (MTT, M2128 SIGMA) for 1 hour. Media was then aspirated, and cells were lysed with dimethyl sulfoxide (DMSO) to solubilize formazan crystals. Absorbance was measured at 570 and 690 nm using UV-Vis spectroscopy via the Cytation 5 Cell Imaging reader (BioTek).

Long Term Survival

Long term growth and clonogenicity was measured using a colony formation assay. Panc02 and MIA PaCa-2 cells were seeded in 6-well plates (1×10^3 cells/well). Cell culture media was replaced every 4 days. After 2 weeks, the resulting colonies were fixed in 1 mL of 100% methanol for 5 minutes and then stained with 5% crystal violet (CV) in 100% methanol for 30 minutes. Plates were rinsed with water, and stained colonies were counted manually.

Exogenous Stress

All cell culture experiments used DMEM and the following conditions were used to modulate nutrient stress *in vitro*:

Table 1. Nutrient-stress media recipes

	Control	**Serum Restriction**	**Glucose Restriction**
FBS	10%	1%	10%
Glucose	25 mM	25 mM	1 mM

Genetic Inhibition of Autophagy: Generation of Atg5 Knockout Cells

To genetically inhibit autophagy, CRISPR/Cas9 technology was used to cleave the gene encoding autophagy protein 5 (Atg5, *Atg5*) from the genome of murine Panc02 cells (Doudna and Charpentier 2014). A lentiviral vector (Sigma-Aldrich) containing genes for both puromycin resistance and green fluorescent protein (GFP) expression was used to transduce the Panc02 cells. Following transduction, cells were selected by culturing in media with puromycin (5 ng/μL). Clonal cell populations were generated by screening for GFP positive single cells.

qPCR was used to confirm the absence of the DNA sequence in the genome. Lack of *Atg5* expression was confirmed through western immunoblotting.

Growth Dynamics

Growth curves were generated by culturing cells under puromycin selection over a 96-hour period. Panc02 $Atg5^{+/+}$ and $Atg5^{-/-}$ cells were seeded in a 24-well plate (4×10^3 cells/well) and cell numbers were analyzed at hours 24, 48, 72, and 96. Cells were trypsinized, stained with trypan blue for live/dead discrimination, and live cells were counted using a hemocytometer.

Three dimensional clonogenic growth was measured using an agarose-based assay. Agarose (0.1%, 2mL) was added to each well of a 6-well dish and left for 30 minutes to solidify. $Atg5^{+/+}$ and $Atg5^{-/-}$ cells (1.0×10^5) were suspended in agarose (0.1%, 7 mL) and culture media (7

mL), and this suspension (2 mL) was added to each well and left to solidify for 30 min. A layer of culture media (2mL) was added to the top of each well and replaced every 4 days.

Extracellular Flux Analyses

Cellular bioenergetics under conditions of nutrient stress were measured using a Seahorse XFe96 Metabolic Analyzer (Agilent Technologies). Panc02 *Atg5*$^{+/+}$ and *Atg5*$^{-/-}$ cells were seeded in a 96-well plate (5 x 10^3 cells/well). After 48 hours, culture media was removed and 100 μL of pre-warmed assay medium (XF base medium supplemented with 5 mM glucose, 2 mM glutamine, 1 mM sodium pyruvate, pH 7.4) was added to each well. The plate was incubated in a CO_2-free chamber at 37°C for one hour. Basal oxygen consumption rate (OCR) was measured.

Invasion and Migration

Matrigel invasion chambers (Corning BioCoat Matrigel Invasion Chambers, #354480) were used to assess invasiveness. Matrigel invasion chambers were thawed for 30 minutes at room temperature and then incubated at 37°C for 2 hours in serum-free media (500 μL). After 2 hours, the Matrigel Invasion Chambers were moved to a fresh plate to wells containing 500 μL of growth factor-supplemented media (10% FBS). Panc02 *Atg5*$^{+/+}$ and *Atg5*$^{-/-}$ cells were seeded in a 6-well plate (1 x 10^4 cells/well) and, after 24 hours, the culture media was replaced with serum-free media for an additional 24 hours. Following serum deprivation, cells were seeded (2.5 x 10^4 cells/assay) into the top chamber of the Matrigel-containing insert. After 18 hours, the Matrigel-containing insert was removed, the underside of the invasion chambers was stained with 5% CV in methanol, and invasive cells were counted.

Migration assays were also used to determine the migratory capacity of *Atg5*$^{+/+}$ and *Atg*$^{-/-}$ Panc02 cells. Cells were seeded (7.0 x 10^4 cells/side of insert) on both sides of an insert (Ibidi #170322/3). After the cells had adhered for 24 hours, the insert was removed, and cell migration

was measured 12 hours after removing the insert via imaging using the EVOS FL Auto 2 Cell Imaging System.

Animal Study

All animal studies were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill. Mice were housed in a climate-controlled Department of Laboratory Animal Medicine facility with a 12-hour light: dark cycle, with *ad libitum* access to water and diets as described below.

Male C57BL/6 mice (N=80) (Charles River Labs, Frederick, MD) were acclimated for a week and then randomly assigned to one of two dietary regimens (n=40/diet). Diets were obtained from Research Diets and dietary regimens included: *ad libitum* control diet (CON) (#D12450J) and a 30% calorie restricted diet (CR) (#D15032801) (**Table 2**).

Table 2. Animal study dietary regimens

Dietary Group	**Regimen**
Control (CON)	Ad libitum, 10 kcal % fat
Calorie Restricted (CR)	30% restriction relative to CON

After 10 weeks on respective diets, mice were sacrificed for intermittent endpoint tissue analysis (n=10/diet) or received intrapancreatic injections of $Atg5^{+/+}$ or $Atg5^{-/-}$ Panc02 cells transduced with a GFP/Luciferase construct (n=15/diet + Panc02 genotype). Weekly imaging was performed following an intraperitoneal injection of 200 µL D-Luciferin followed by isoflurane anesthesia using an IVIS Lumina II. In accordance with humane euthanasia regulations, mice were euthanized at the 14-week time point using carbon dioxide inhalation and cervical dislocation. Tumor, pancreas, muscle, liver, and serum were collected from each mouse.

Results:

I. Pharmacological Inhibition of Autophagy

In vitro glucose restriction induces autophagy in pancreatic cancer

Nutrient stress in KRAS-driven PDAC induces autophagy, a process that has been associated with promoting tumor growth and progression due to mobilization of metabolites used to provide energy (Guo, Chen et al. 2011, White 2015). We hypothesized that pharmacological inhibition of autophagy under conditions of nutrient stress would induce apoptosis and impair colony formation of PDAC cell lines *in vitro*. Under nutrient replete conditions, CQ treatment resulted in significantly greater LC3B II accumulation compared to vehicle treatment in MIA-Paca-2 cells indicating the disrupted maturation of the autophagolysosome, and therefore inhibition of autophagy (Figure 1B; P= 0.015 a v b). In Panc02 cells, CQ treatment, relative to control, resulted in moderately increased LC3B II accumulation that did not reach statistical significance (Figure 1A; P=0.094).

Serum restriction and glucose deprivation were used to induce nutrient stress *in vitro* (Table 1**,** Methods). Under the culture conditions used, neither serum deprivation or glucose restriction induced autophagy as measured by LC3B II accumulation in Panc02 and MIA Paca-2 cells (Figure 1A, 1B). However, CQ administration in combination with glucose restriction in Panc02 cells resulted in significantly greater LC3B II accumulation compared to the 10% FBS control and the 1% FBS condition (Figure 1A; P=0.039 a v b, P=0.048 a v b). These findings suggest that CQ treatment effectively inhibits autophagy induced by glucose restriction.

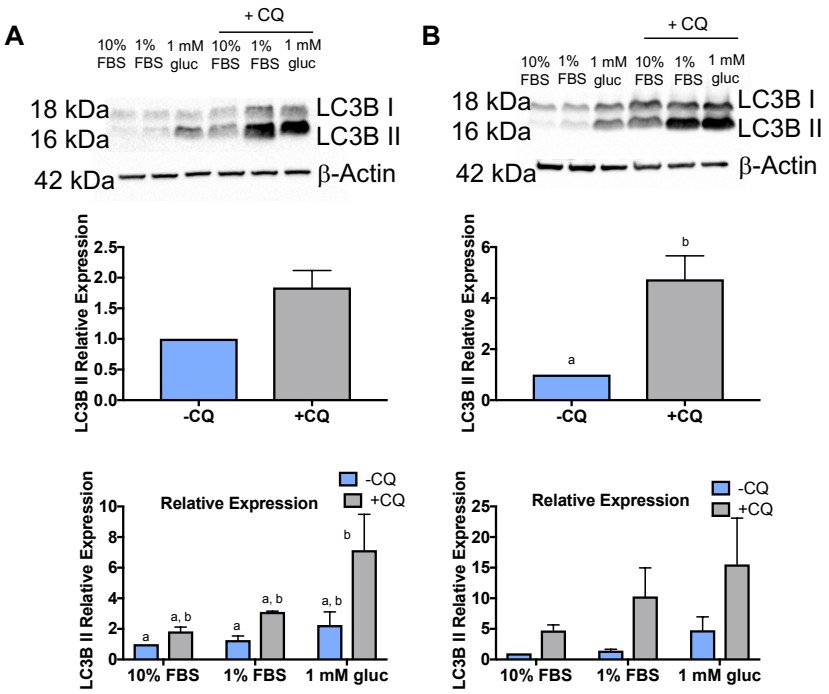

Figure 1. Autophagy induced by reduced FBS or glucose can be inhibited by CQ
Panc02 cells **(A)** and MIA PaCa-2 cells **(B)** were treated with 10 μM CQ for 48 hours and lysed. LC3B protein expression was analyzed. Representative images selected from N=3 experiments. Data presented as the mean ± SEM. An unpaired t-test was used for data sets containing two groups, two-way ANOVA with Sidak's multiple comparison test was used for data analysis of multiple groups ($P < 0.05$ was considered statistically significant, different letters indicate significance).

Autophagy inhibition in combination with growth factor reduction drastically impairs proliferation in pancreatic cancer cells

In Panc02 cells, culture in low-serum conditions resulted in significant reduction in proliferation of approximately 50% relative to control (Figure 2A; P<0.0001 a vs b), with similar degree of proliferation inhibition observed under conditions of glucose restriction (Figure 2B; P=0.049 a vs b). The short-term application of CQ in growth factor-replete media also significantly reduced Panc02 proliferation relative to control, while the application of CQ in the context of low serum further inhibited proliferation (Figure 2A; P<0.0001 a vs c, P<0.0001 b vs c). In contrast, short-term CQ addition in the context of low glucose did not exert any additional inhibitory effect on Panc02 proliferation (Figure 2B). Low serum conditions also impaired Panc02

colony formation, a measure of proliferation on a longer time scale, to a greater extent than low-glucose in the absence of CQ (Figure 2C; P<0.0001 a vs c, P<0.0001 a vs d). Remarkably, long term *in vitro* administration of CQ substantially impaired Panc02 colony formation under all conditions (Figure 3C; P<0.0001 a vs b). While the effects of serum and glucose restriction on proliferation in MIA PaCa-2 cells were less pronounced (Figures 3D-E), chronic treatment with CQ again drastically impaired colony formation under all culture conditions (Figure 3F; P<0.0001 a vs b). These results strongly support a role for autophagy in survival of PDAC lines, and that autophagy induction plays a key role in cellular to nutrient stress.

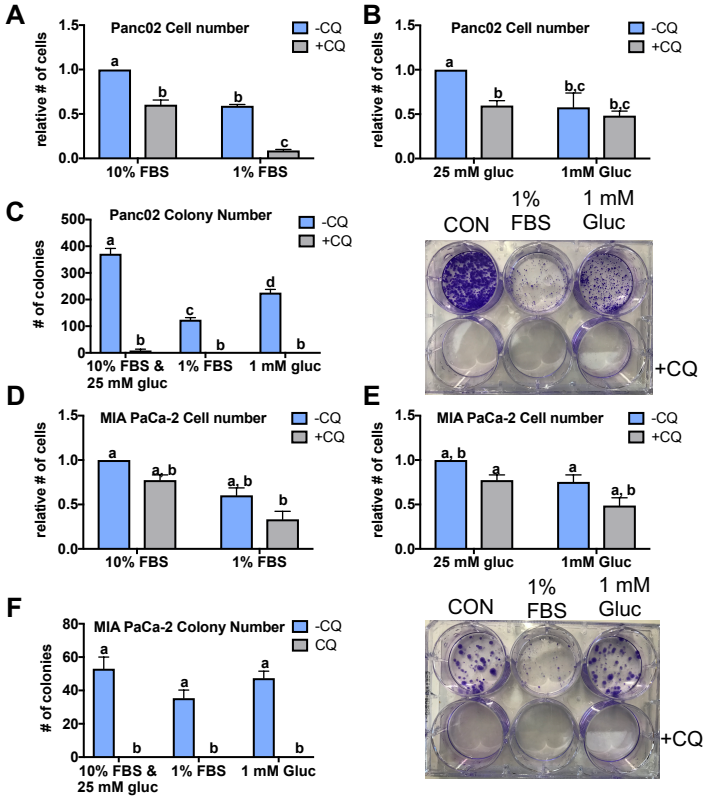

Figure 2. CQ and nutrient restriction synergize in PDAC growth inhibition
Panc02 cells **(A, B)** and MIA PaCa-2 cells **(D, E)** were treated with nutrient deplete media and 10 μM CQ for 48 hours and an MTT assay was used to measure viability. Additionally, Panc02 cells **(C)** and MIA PaCa-2 cells **(F)** were treated with nutrient deplete media and 10 μM CQ for 2 weeks with treatment media changed every 4 days; colonies were stained with crystal violet. Representative images selected from N=3 experiments. Data presented as the mean ± SEM. Two-way ANOVA with Tukey's multiple comparison test was used for data analysis of multiple groups ($P < 0.05$ was considered statistically significant, different letters indicate significance).

II. Genetic Autophagy Deficiency

Permanent loss of Atg5 does not impair PDAC proliferation in 2-dimensional culture

While CQ is effective for transiently inhibiting autophagy, we hypothesized that permanent autophagy ablation over time may result in the development of autophagy independence in PDAC cells. To investigate the effect of permanent, total autophagy inhibition on viability of PDAC cells, CRISPR/Cas9 was used to generate a Panc02 cell line deficient in *Atg5*. Western immunoblot analysis revealed no detectable expression of Atg5 at 55 kDa, and the lack of LC3BII at 16 kDa demonstrates prevention of LC3B lipidation and therefore disruption of autophagosome formation (Figure 3A). Despite autophagy deficiency in the $Atg5^{-/-}$ cells, no significant differences in short term proliferation as measured through the expression of PCNA, 96-hour growth, or MTT assay (Figure 3A, 3B, 3C). In stark contrast to the results observed with chronic pharmacological inhibition (Figure 2C & 2F), long term clonogenic growth was also unaffected in $Atg5^{-/-}$ cells compared to $Atg5^{+/+}$ cells (Figure 3D). Thus *in vitro* results indicate that permanent autophagy inhibition in the Panc02 PDAC line does not alter growth dynamics under adherent conditions which indicates profound cellular reprogramming.

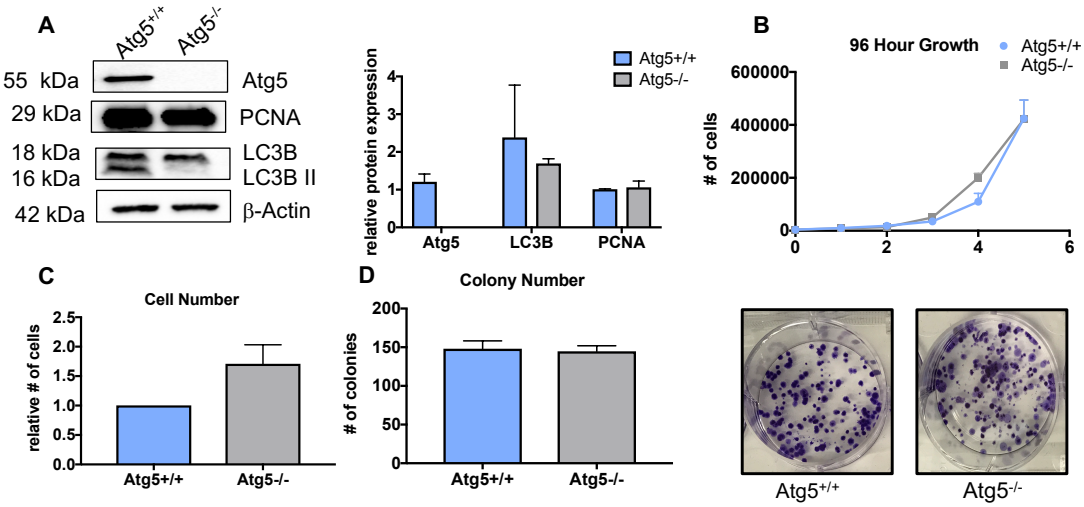

Figure 3. *Atg5* knockout in Panc-02 cells does not affect growth *in vitro*
Atg5$^{+/+}$ and *Atg5*$^{-/-}$ cells were lysed and expression of Atg5, PCNA, and LC3B were analyzed **(A)**. Short term growth was measured counting *Atg5*$^{+/+}$ and *Atg5*$^{-/-}$ cells over a 96-hour period **(B)**. *Atg5*$^{+/+}$ and *Atg5*$^{-/-}$ cells were seeded and after 48 hours, an MTT assay was used to measure viability **(C)**. *Atg5*$^{+/+}$ and *Atg*$^{-/-}$ cells were seeded and media was changed every four days; colonies were stained with crystal violet. Representative images selected from N=3 experiments. Data presented as the mean ± SEM. An unpaired t-test was used for data sets containing two groups, two-way ANOVA with Sidak's multiple comparison test was used for data analysis of multiple groups ($P < 0.05$ was considered statistically significant, different letters indicate significance).

Consistent with results shown in Figure 2, growth in serum restricted conditions significantly reduced proliferation of *Atg5*$^{+/+}$ by more than 50% relative to control conditions (Figure 4A; P<0.0001 a vs b). However, glucose restricted media did not exert any inhibitory effect on *Atg5*$^{+/+}$ proliferation (Figure 4D). In nutrient replete conditions, *Atg5* knockout did not alter the rate of proliferation compared to wildtype cells (Figure 4A, 4D). In contrast, under serum-restricted conditions the proliferation of *Atg5*$^{-/-}$ was significantly impaired; notably, *Atg5*$^{+/+}$cells were more sensitive to serum depletion compared to Atg5$^{-/-}$ cells (Figure 4A; P=0.0008 a vs c, P<0.0023 b vs c). Similar to our findings with transient autophagy inhibition (Fig 2B and 2E), in the context of low glucose, deletion of *Atg5* in PDAC did not affect cell proliferation (Figure 4D). In *Atg5*$^{+/+}$ cells, low serum conditions diminished *Atg5*$^{+/+}$ colony formation to a greater extent than

low glucose (Figure 4B; P=0.0008 a vs b, Figure 4E; P=0.0498 a vs b). Importantly, 2D colony formation was significantly less impaired by serum deprivation and glucose restriction in *Atg5*$^{-/-}$ cells compared to *Atg5*$^{+/+}$ (Figure 2B; P=0.0046, Figure 2E; 0.0498). To analyze differential metabolism occurring as a result of Atg5 knockout, oxygen composition rates (OCR) were measured. Neither nutrient stress nor autophagy inhibition had a significant effect on basal OCR (Figure 4C & F). These results suggest that permanent autophagy ablation, as occurs with the Atg5 knockouts, does not further hinder growth in the context of nutrient stress, but rather that an adaptive response to sustained loss of autophagy exerts a protective effect in PDAC.

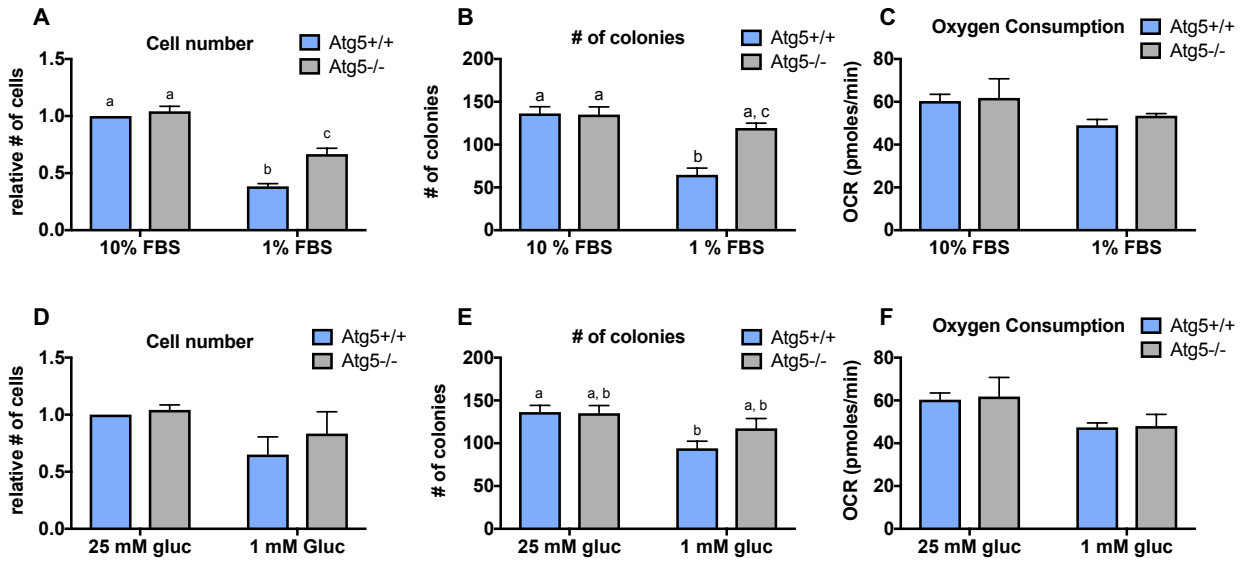

Figure 4. Effects of exogenous nutrient stress and autophagy deficiency on cell growth and metabolism *Atg*$^{+/+}$ and *Atg*$^{-/-}$ cells were treated with serum-restricted media **(A)** and low-glucose media **(D)** for 48 hours and an MTT assay was used to measure viability. Additionally, *Atg*$^{+/+}$ and *Atg*$^{-/-}$ cells were treated serum-restricted media **(B)** and low-glucose media **(E)** for 2 weeks with treatment media changed every 4 days; colonies were stained with crystal violet. Oxygen consumption was measured under serum restricted **(C)** and low glucose conditions **(F)**. Data presented as the mean ± SEM for N=3 experiments. Two-way ANOVA with Tukey's multiple comparison test was used for data analysis of multiple groups ($P < 0.05$ was considered statistically significant, different letters indicate significance).

Atg5 deficiency reverses phenotypes associated with EMT and inhibits anchorage independent growth

An anchorage-independent, agarose-based assay was used to monitor three-dimensional growth. This assay revealed that *Atg5*$^{+/+}$ cells form colonies more readily in 3D culture compared to *Atg5*$^{-/-}$ cells (Figure 5A; P<0.0001). Anchorage-independent growth has been associated with increased metastatic potential of cancer cells (Lyons, Alizadeh et al. 2016). First, we measured markers of epithelial-to-mesenchymal transition in *Atg5*$^{+/+}$ and *Atg5*$^{-/-}$. *Atg5* deletion did not significantly alter expression of the epithelial marker E-Cadherin, but significantly decreased the expression of the mesenchymal markers N-Cadherin and Snail in *Atg5*$^{-/-}$ cells (Figure 5A; P=0.033 a v b, P=0.0017 a v b). Additionally, Snail expression in *Atg5*$^{-/-}$ cells appears at a higher molecular weight, and is likely highly phosphorylated which further indicates the degradation and inactivation of this protein. Furthermore, *Atg5* deletion resulted in significant increases in cell migration, yet a significant and substantial decrease in invasiveness (Figure 5B; P=0.006, Figure 5C; P=0.001). Migration enables cells to translocate, while invasion refers to movement through the extracellular matrix (ECM). These findings suggest that the development of autophagy independence influences EMT via reduced cellular plasticity through the robust selective pressure exerted on these cells.

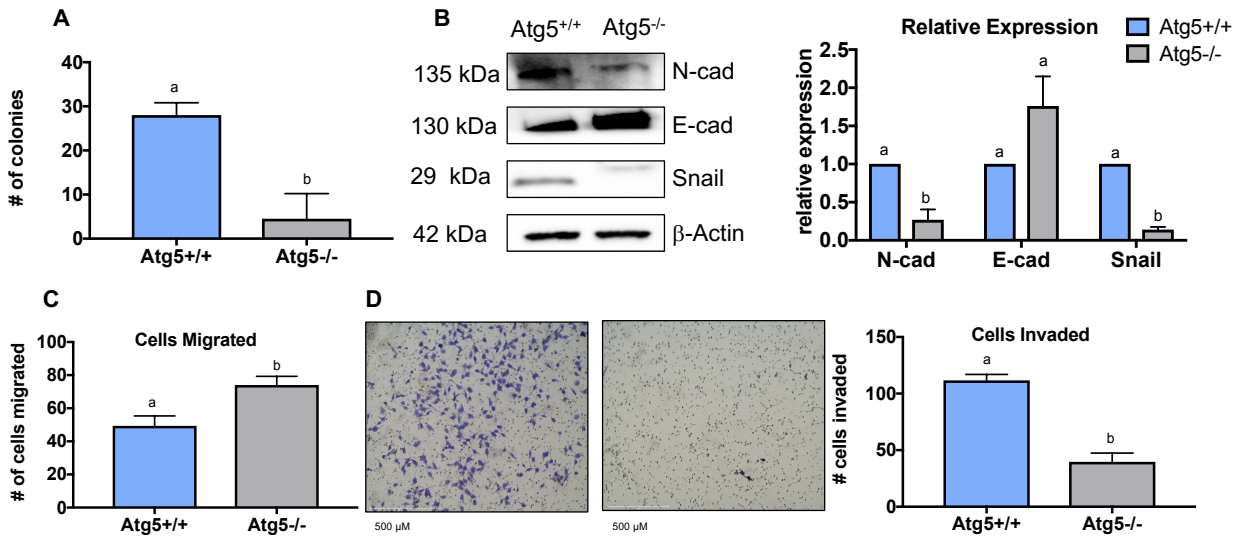

Figure 5. Atg5 deletion induced a more epithelial-like phenotype
Cells were grown in an agar-based assay for 2 weeks and formed 3D colonies **(A).** Markers of EMT including N-Cadherin, E-Cadherin, and Snail were altered with *Atg5* knockout and quantified **(b).** Cell migration was significantly increased in *Atg5*$^{-/-}$ cells **(C)**, while invasion was significantly decreased **(D).** Data presented as the mean ± SEM, n=3, representative images selected. An unpaired t-test was used for data analysis ($P < 0.05$ was considered statistically significant, different letters indicate significance)

Animal Study

Our *in vitro* studies above indicated that permanent autophagy ablation interferes with anchorage-independent growth yet may also allow for resilience in the face of nutrient stress. To determine whether our *in vitro* findings influenced PDAC biology *in vivo*, cells were transplanted into an animal model of caloric restriction (CR). At the time of sacrifice, male C57BL/6 mice prescribed a CR diet had significantly decreased body weight compared to mice on a CON diet (P<0.0001). Upon intrapancreatic injection, the complete loss of autophagy in *Atg5*$^{-/-}$ cells generated smaller tumors in mice prescribed a CON and CR diet compared to control *Atg5*$^{+/+}$ cells (Table 2, Methods, Figure 6B; P<0.0001 a v b; P=0.044 b v c). Additionally, the CR dietary regimens did not have a significant effect on tumor weight compared to the CON diet regardless of the Atg5 status of the cells. While *Atg5* deficiency did not affect growth *in vitro*, these results

show that cellular adaptation to loss of autophagy profoundly alters the tumorigenicity of these cells.

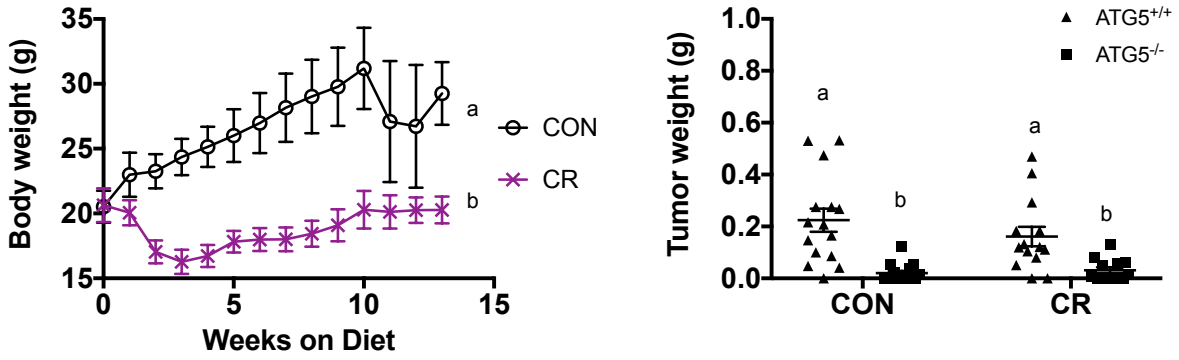

Figure 6. *Atg5* deficiency significantly impaired tumor formation
Mice were prescribed a CON or CR dietary regimens for the course of the study resulting in differential body weights **(A).** Tumor weight **(B)** was measured at time of sacrifice. Data presented as the mean ± SEM, N=15 per group. Two-way ANOVA with Tukey's multiple comparison test was used for data analysis of multiple groups ($P < 0.05$ was considered statistically significant, different letters indicate significance).

Discussion:

This body of work characterizes the effects of transient versus permanent inhibition of autophagy in PDAC in combination with nutrient stress. We first confirmed inhibition of autophagy in PDAC cells in response to CQ. While transient CQ treatment did not have a significant effect in Panc02 cells, LC3B II accumulation was observed in MIA PaCa-2 cells, indicating successful inhibition of autophagy (Figure 1). A greater number of Panc02 experimental replicates would likely also show significant autophagy inhibition in response to CQ treatment. Interestingly, while the mechanistic inhibition and induction of autophagy was not significant across cell lines, both transient and chronic CQ treatment and nutrient stress drastically reduced Panc02 and MIA PaCa-2 cell proliferation (Figure 2). Perhaps more integrated measures of autophagy flux using different parameters such as a dual tag mCherry-GFP construct would better quantify this dynamic process (Bjørkøy, Lamark et al. 2009) and confirm the connection between nutrient stress, autophagy, and the observed inhibitory effect of CQ on PDAC growth *in vitro*.

Nutrient stress was induced *in vitro* through serum restriction and glucose restriction. Of these two stressors, serum restriction consistently had more pronounced effects on growth inhibition in the presence and absence of CQ. The powerful effects of serum restriction are likely the result of alterations in a variety of pro-growth signaling pathways. For example, growth factor restriction has previously been shown to reduce the activity of the Ras/MAPK and PI3K/Akt/mTOR pathways, leading to decreased expression of genes involved with proliferation (Jiang, Zhu et al. 2008). In all growth conditions, and across both cell lines, the most substantial reduction in growth was observed with chronic CQ treatment over a two-week period, where long-term CQ treatment nearly eliminated growth of PDAC cells (Figure 2 C & F).

However, a small number of colonies did survive, possibly due to drug efflux, desensitization, or development of autophagy independence in response to prolonged treatment with CQ. Thus, we next investigated the effects of permanent autophagy inhibition in Panc02 cells to identify the impact of nutrient restriction in an autophagy-independent PDAC cell line.

Permanent autophagy inhibition was analyzed through the genetic deletion of *Atg5* in Panc02 cells. *Atg5* was selected because the protein product of *Atg5* is required for formation of the autophagosome. Contrary to the differential growth observed with transient autophagy inhibition, permanent autophagy inhibition had insignificant effects on short and long-term, two-dimensional growth (Figure 3). $Atg5^{+/+}$ cells were also more sensitive to nutrient stress than $Atg5^{-/-}$ cells (Figure 4). Perhaps the permanent absence of autophagy in *Atg5* knockout cells led to the upregulation of alternate metabolic pathways as compensatory mechanisms, yielding cells that were no longer reliant on autophagy (Ding, Chen et al. 2012). Alternatively, Nishida *et al.* showed that macroautophagy can be induced via a separate, *Atg5*-independentpathway (Nishida, Arakawa et al. 2009). Additionally, nuclear factor erythroid 2 (Nrf2) signaling has been shown to be induced under conditions of autophagy deficiency, while proline metabolism may also be upregulated to provide energy in the absence of autophagy (Phang, Pandhare et al. 2008, Lau, Wang et al. 2010, Gurley, Ilkayeva et al. 2016). Further investigation of autophagic flux and amino acid metabolism would reveal if *Atg5* independent autophagy was indeed occurring in $Atg5^{-/-}$ cells (Nishida, Arakawa et al. 2009). Studies are underway in the Hursting Lab to establish the mechanisms underlying the metabolic reprogramming that occurs with permanent Atg5 ablation.

Notably, three dimensional clonogenic growth revealed stark differences in the ability of $Atg5^{+/+}$ and $Atg5^{-/-}$ cells to form colonies. $Atg5^{+/+}$ cells formed colonies more readily under anchorage-independent conditions. These findings were consistent with decreased expression of

the mesenchymal markers N-Cadherin and Snail in *Atg5*$^{-/-}$ compared to *Atg5*$^{+/+}$ cells (Figure 5). Interestingly, we also created *Atg5* deficient lines in two additional claudin-low triple negative breast cancer cell lines (M-Wnt and met M-Wnt^{lung} cells), both of which showed similar modification in EMT markers. To investigate the impact of the reduced expression of EMT-related proteins, cell migration and invasion were measured. Cellular invasion was decreased in the epithelial-like, *Atg5*$^{-/-}$ cells; however, migration was increased (Figure 5). The increased migration of the *Atg5*$^{-/-}$cells may be a result of increased cell-cell interactions that facilitate migratory rates (Schaeffer, Somarelli et al. 2014) or differential expression of proteases involved in remodeling of the extracellular environment. Integrins can detect environmental changes and relay information to alter cell shape, adhesion, and migration; proteases are released during invasion and can help remodel the ECM and facilitate movement into the stroma (Hood and Cheresh 2002, Yamaguchi and Condeelis 2007). Therefore, additional experiments could investigate integrin and protease activity in order to better understand the differences in cell migration and invasion in *Atg5*$^{+/+}$ and *Atg5*$^{-/-}$ cells.

Finally, to study the *in vivo* relevance of autophagy dependency under conditions of caloric restriction (nutrient stress), *Atg5*$^{+/+}$ and *Atg5*$^{-/-}$ cells were used in an animal model. Male C57BL/6 mice were used because the Panc02 cell line was derived from the C57BL/6 mouse strain, and PDAC incidence is higher in men (Wang, Shi et al. 2001, Siegel, Miller et al. 2018). In order to impose nutrient stress *in vivo*, a CR diet was prescribed and reduced caloric intake by 30% (Table 2, Methods). Our findings herein match those of Lashinger *et al.* in showing that permanent autophagy inhibition decreases tumor burden (Lashinger, O'Flanagan et al. 2016), as *Atg5* deficiency significantly impaired tumor formation and growth in both CON and CR conditions. However, the results of Lashinger *et al.* showed that CR in a PDAC model decreased tumor volume

and progression (Lashinger, O'Flanagan et al. 2016). Our findings did not indicate any effect of CR on tumor size, likely because the tumors our study were much smaller than those reported by Lashinger et al.

Reduced tumor growth by autophagy-deficient cells is consistent with our results from the anchorage independent growth assays, and suggest that *Atg5*-dependent autophagy plays a crucial role in tumor development in three dimensions (Figure 5&6). In light of the EMT related findings, repeating our animal study using a highly metastatic cell line and analyzing the relative number of metastases would contribute to understanding the relationship between EMT and autophagy *in vivo*.

PDAC continues to be a leading cause of cancer related deaths and remains a pressing public health issue (Siegel, Miller et al. 2018). In order to improve PDAC outcomes, a more complete understanding of the metabolic weaknesses of PDAC cells may allow for novel interventions. Autophagy inhibition has been used in isolation as an adjuvant therapy; however, our findings suggest that use in combination with nutrient stress may be more effective (Wolpin, Rubinson et al. 2014). Additionally, we posit that prolonged transient autophagy inhibition may select for cells that are not reliant on autophagy. Therefore, understanding and targeting metabolic differences between autophagy-independent cells and autophagy-competent cells may facilitate the development of more effective treatment strategies for Kras-driven pancreatic cancer.

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