Acknowledgements

I would like to thank Dr. Channing Der for giving me the opportunity to learn in his lab, and his full support throughout my years in the lab. I would like to thank Dr. Kirsten Bryant for spending countless hours training me, challenging me, and expecting the most of me from day one. And I would like to thank my parents, who continuously remind me that I can achieve anything if I am willing to work for it.

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I. ABSTRACT

Background: Pancreatic cancer has a dismal 5-year survival rate of 9%. *KRAS* is mutated in 98% of pancreatic ductal adenocarcinomas (PDACs), making the discovery of a treatment for *KRAS* mutant cancers urgent. However, over 30 years of effort have yet to yield a direct anti-KRAS therapeutic, thus a current focus of the field is targeting downstream effectors of mutant *KRAS*.

Hypothesis: We hypothesize that the synergy seen between SCH772984 (SCH), an ERK inhibitor, and chloroquine, an inhibitor of lysosomal acidification, is due to the ability of chloroquine to block the cell from harnessing energy through autophagy.

Methods: HPAC, PANC-1, Pa14C and Pa01C cell lines were treated with a combination of SCH and autophagy specific inhibitors Spautin-1, SBI-0206965 (SBI), and MRT68921 (MRT). Cells were treated with the drugs alone and in combination with SCH for 72 hours, and proliferation assays were used to quantify the effect on cell growth. In a parallel line of investigation, ATG5 and ATG7 were genetically silenced using shRNA in a panel of PDAC cell lines. Following western blot analyses to confirm ATG5 or ATG7 knockdown, the sensitivity of these cells to SCH were compared to that of parental cells using proliferation after 72 hours of treatment as a readout.

Results: Each autophagy inhibitor analyzed synergized with SCH in multiple PDAC cell lines. For these studies, the autophagy inhibitors were held at constant concentrations, while the SCH concentration was varied. Each of the drugs displayed the most synergy

with SCH at concentrations ranging from 0.156μM to 1.25μM. MRT consistently showed the most synergy in our studies. In addition, growth curves of PDAC cells with ATG5 or ATG7 knockdown demonstrated that genetic silencing of autophagy-related proteins increase sensitivity to SCH treatment compared to wild type. PDAC cells became more sensitive to SCH following each of these forms of autophagy inhibition in our studies.

Conclusion: Each of the autophagy inhibitors synergized with the ERK inhibitor in multiple PDAC cell lines. Additionally, cells in which vital autophagy genes, ATG5 or ATG7, were knocked down were more sensitive to SCH. These findings suggest that the synergy between chloroquine and SCH is at least partially due to the inhibition of autophagy.

II. INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest cancers with a 9%, 5-year survival rate, and it is projected to become the second leading cause of cancer death by 2020 (Rahib et al., 2014). Clinically, symptoms of PDAC include jaundice, weight loss, poor appetite, nausea, and stomach or back pain (Siegel, Miller, & Jemal, 2017). Due to difficulty in distinguishing these symptoms from other diagnoses, and the lack of a sufficient clinical diagnostic tool, PDAC is typically not detected until it reaches later stages. Currently, the lone curative option is surgery, but only 20% of patients are eligible because most patients present with metastatic disease and do not qualify for surgery. Thus, many patients are in need of new treatments.

The three *RAS* genes (*HRAS*, *NRAS*, and *KRAS*) modulate cellular differentiation, proliferation, migration and apoptosis making them essential for cellular signaling. RAS is a GTPase that is activated when it is bound to guanosine triphosphate (GTP) and inactive when it is bound to guanosine diphosphate (GDP). RAS GTP-GDP cycling is regulated by guanine nucleotide exchange factors (GEFs) that stimulate nucleotide exchange and by GTPase activating proteins (GAPs) which accelerate GTP hydrolysis activity. Once activated, RAS-GTP interacts with numerous signaling pathways that regulate diverse cellular processes. Mutation of residues G12, G13 and Q61 prevent the association between KRAS and GAPs by reducing van der Waals interactions (Scheffzek et al., 1997) and also interfere with the water molecule necessary for GTP hydrolysis (Scheidig, Burmester, & Goody, 1999). The *RAS* genes are the most frequently mutated oncogenes in human cancers. *KRAS* is mutated in 98%

of PDAC, and is an accepted driver of PDAC growth and maintenance (Cox, Fesik, Kimmelman, Luo, & Der, 2014).

Because KRAS drives PDAC, there have been many efforts to target this protein using a variety of mechanisms (Papke & Der, 2017). Targeting the protein directly has been especially difficult, because RAS signals through protein-protein interactions (Tanaka & Rabbitts, 2010), which are difficult to target with small molecules. This has resulted in a shift in focus to downstream effectors of KRAS. Most efforts have concentrated on the RAF-MEK-ERK (MAPK) and the PI3K-AKT-mTOR pathways. There are three isoforms of RAF; ARAF, BRAF and CRAF. There are currently two BRAF inhibitors approved to treat *BRAF*-mutant melanoma, vemurafenib and dabrafenib. Unexpectedly, these inhibitors activate rather than inhibit ERK in *RAS*-mutant cancers (Hatzivassiliou et al., 2010; Heidorn et al., 2010). MEK inhibitors have been more successful, and have shown greater effect when used in combination with BRAF inhibitors as opposed to being used as a monotherapy (Flaherty et al., 2012). This is thought to be due to compounded dampening of the pathways that lead to ERK reactivation. Despite some inhibitors reaching the clinic, they have been largely unsuccessful in RAS-mutant cancers as monotherapies. Resistance to BRAF and MEK inhibitors monotherapy is due to upregulation of upstream activators that reactivate ERK (Ryan, Der, Wang-Gillam, & Cox, 2015). There are four ERK inhibitors that have entered Phase 1 clinical studies (BVD-523, MK8353, GDC-0994, and CC-90003), and MK8353 (analog of SCH772984) has been described preclinically (Morris et al., 2013). A recent study from our lab has showed that mechanistically ERK inhibition leads to MYC degradation and the initiation of a senescence like phenotype (Hayes et al., 2016).

This study also suggested that PI3K-AKT-mTOR signaling plays an important role in ERK inhibitor sensitivity and that combination treatments will be the most successful in treating PDAC patients (Hayes et al., 2016).

It has been demonstrated that *KRAS* mutant cancer cells alter their metabolic programming to better suit their rapid proliferation; thus, another recent approach to targeting KRAS has been to target the metabolic processes it regulates (Bryant, Mancias, Kimmelman, & Der, 2014). *KRAS*-driven metabolic alterations include induction of glucose uptake, altered glutamine metabolism and an upregulation of macropinocytosis and autophagy. Autophagy is a mechanism utilized by the cell to recycle damaged organelles and provide ATP and amino acids, lipids, sugars and nucleosides (Rabinowitz & White, 2010). Autophagy is induced by nutrient depletion or starvation; subsequently, an autophagosome will form, sequestering cytosolic proteins and organelles by a double-membrane vesicle. The autophagosome will then fuse with lysosomes, which will breakdown the contents of autophagic vesicle and release macromolecules for the cell to reuse.

While it appears that PDAC cells rely on autophagy for growth, the role of KRAS in this upregulation is still not completely understood. PDAC cell lines display elevated levels of autophagy (S. Yang et al., 2011). *In vitro* studies show chloroquine, an indirect inhibitor of autophagy, was more effective in the treatment of PDAC cell lines with elevated autophagy, compared to non-PDAC cell lines with low basal autophagy (H460 and MCF7) (S. Yang et al., 2011). Although an *in vivo* study suggested that chloroquine might be tumor promoting in RAS-dependent pancreatic cancers that develop with homozygous p53 mutations (Rosenfeldt et al., 2013), other studies have attributed this

result to the homozygous deletion of p53, and support using anti-autophagy therapies in PDAC (A. Yang et al., 2014). Chloroquine was unsuccessful as a monotherapy in clinical trials (Wolpin et al., 2014), but autophagy inhibition alone may not be sufficient to prevent tumor growth, whereas a combination treatment may be successful.

In an effort to determine whether the efficacy of an ERK inhibitor could be enhanced with the additional targeting of a second pathway, a previous graduate student in the lab collaborated with Krister Wennerberg (Institute for Molecular Medicine, Finland) to determine what drugs synergize with ERK and MEK inhibitors. The drug screen was done in the PDAC cell lines HPAC and Panc10.05. Cells were exposed to dose-dependent drug sensitivity testing against 309 compounds in the presence and absence of the ERK inhibitor, SCH772984, or the MEK inhibitor, AZD6244 (Hayes et al., 2016). Interestingly, chloroquine, and inhibitor of lysosomal acidification, synergized with SCH772984. Chloroquine accumulates in the acidic parts of the cell, like the lysosome, and leads to the inhibition of enzymes that require an acidic pH, thus preventing lysosomal fusion (Hsin et al., 2012). Because chloroquine prevents the fusion of the autophagosome to the lysosome, it indirectly inhibits autophagy. However, the cell degrades other remnants and endosomes, including macropinosomes via the lysosome. Thus, it is unclear whether the effect of chloroquine in combination with SCH772984 is due to the inhibition of autophagy in particular, or if it is a more general effect of the inhibition of lysosomal acidification.

III. SPECIFIC AIMS, HYPOTHESIS

PDAC remains one of the deadliest cancers with a 5-year survival rate of 9% (Rahib et al., 2014). It remains difficult to detect due to its general symptoms and the lack of any accurate diagnostic tools to detect the cancer before it has spread. Current treatments for metastatic PDAC are minimally effective, and there remains a dire need for new therapies.

The commitment to finding an anti-RAS drug has yet to yield any effective clinical treatments, resulting in many labeling the protein “undruggable”. But the field is adapting, and finding new approaches to target RAS mutant cancers. One approach involves preventing RAS from attaching to the plasma membrane. RAS must be positioned on the inner face of the plasma membrane to be active, so many have attempted to prevent this attachment by targeting post translational modifications that are attached to the CAAX motif. Our lab has mostly focused on targeting downstream effectors of RAS, the most validated pathways are the PI3K-AKT-mTOR and RAF-MEK-ERK pathways. Targeting the RAF-MEK-ERK signaling cascade has yielded some clinical trial candidates, which have ultimately failed as monotherapies in PDAC. We are currently focusing on the ERK inhibitor due to recent data suggesting that it may be an effective treatment when used in combination with other drugs (Hayes et al., 2016). A new and novel approach for treating RAS mutant cancers is by targeting metabolic processes. We have specifically focused on autophagy due to its clear upregulation in PDAC (Guo et al., 2011; S. Yang et al., 2011). Autophagy is a scavenging process that results in the recycling of damaged organelles and proteins to produce energy for the cell (Figure 1). Autophagosomes, containing the remnants of the cell that will be

degraded, are broken down by the lysosome. Chloroquine is an inhibitor of lysosomal acidification, and inhibits autophagy by preventing the autophagosome from fusing with the lysosome. Chloroquine has not been successful as a monotherapy in humans; however, combination therapy with other drugs is still under investigation (Wolpin et al., 2014).

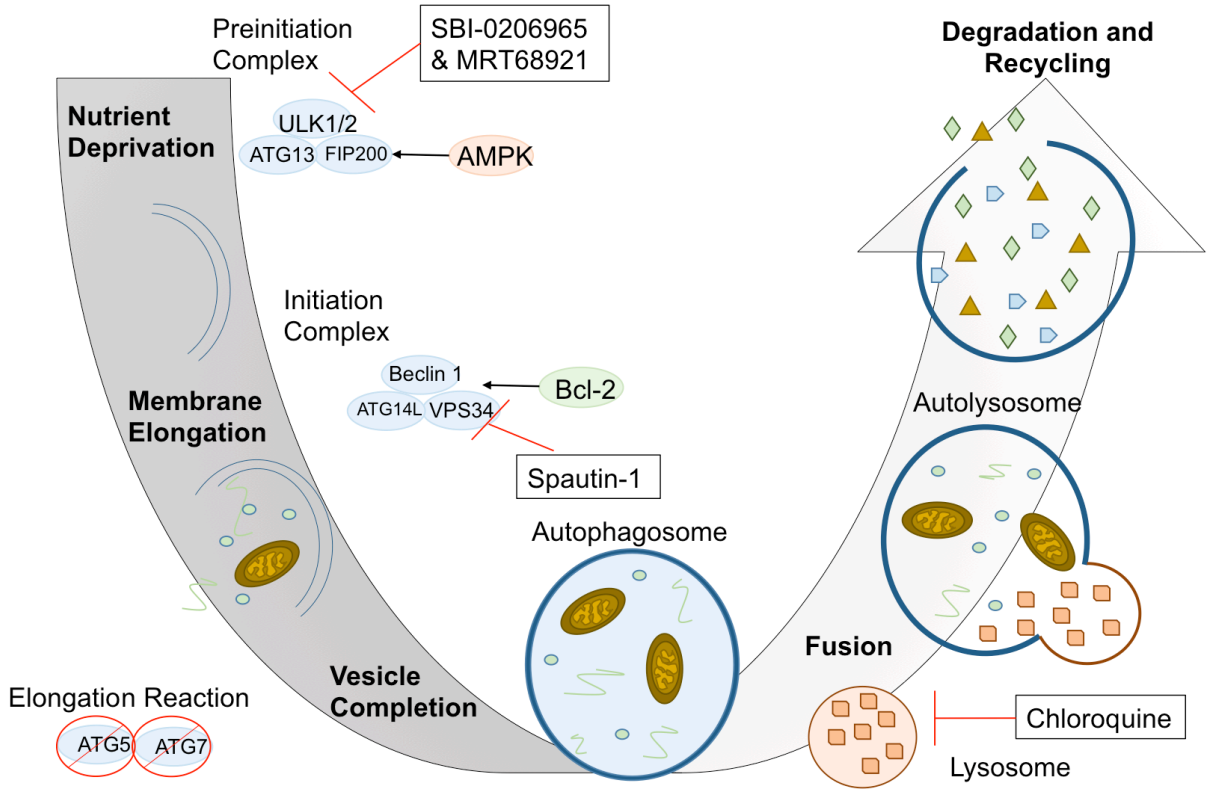

Figure 1. The metabolic process of autophagy. Autophagy is initiated by AMPK when cells are deprived by energy. The membrane of the autophagosome is elongated and surrounds proteins and organelles that will be recycled. The autophagosome fuses with the lysosome to form an autolysosome, which contains the degraded remnants of the cell. Those remnants are then released into the cytoplasm. The approaches taken in our studies targeted different stages of this process. SBI-0206965 inhibits ULK1 and MRT68921 inhibits the ULK1/2 complex, which are part of the preinitiation complex. Spautin-1 inhibits VPS34, part of the initiation complex. Knockdown of ATG5 and ATG7 targets the elongation reaction of the membrane so it cannot be completely formed.

Unpublished data from our laboratory demonstrated that KRAS mutant pancreatic cancer cells exhibit upregulated autophagic flux following ERK inhibition and KRAS knockdown (Bryant, KL). Furthermore, in collaboration with Krister Wennerberg, (FIMM, Finland) we showed that an inhibitor of autophagy, chloroquine, synergized with

the ERK inhibitor in multiple pancreatic cancer cell lines (Hayes et al., 2016). Dr. Bryant validated the synergy in multiple PDAC cell lines using two chemically distinct ERK inhibitors. However, because chloroquine has is an inhibitor or lysosomal acidification and does not directly inhibit autophagy, we aimed to validate whether the synergistic effects are due to the inhibition of autophagy or the more general inhibition of the lysosome. In our Aim 1 studies, we will utilize specific inhibitors of autophagy and determine if they too synergize with ERK inhibition to slow PDAC cell proliferation. We will utilize two different inhibitors of ULK, an upstream regulator of autophagy, SBI-0206965 (ULK1) and MRT68921 (ULK1/2), as well as Spautin-1, an inhibitor of a further downstream regulator, Vps34 (Figure 1). While these inhibitors are not clinical candidates, like chloroquine, they more directly inhibit the process of autophagy and can be used in cell culture experiments. In my Aim 2 studies we will utilize a genetic approach, shRNA-mediated knockdown of genes essential for autophagy (ATG5 and ATG7), to assess whether cells that are deficient in autophagy are more sensitive to ERK inhibition (Figure 1). We hypothesize that the effect of chloroquine, in combination with SCH, is at least partially due to the inhibition of autophagy, which we will determine using different forms of autophagy inhibition in combination with SCH.

Aim 1: To determine whether the autophagy inhibitors SBI-0206965, MRT68921, and Spautin-1 synergize SCH, and whether the level of synergy is comparable to that of chloroquine with SCH. We will evaluate the effects of each autophagy inhibitor on the proliferation of a panel of human PDAC cells and then treat cells with SCH alone

and in combination with each autophagy inhibitor to determine if we see synergy between these drugs and ERK inhibition.

Aim 2: To establish whether PDAC cells in which ATG5 or ATG7 has been genetically silenced are more sensitive to SCH treatment. We will obtain five shRNAs for either ATG5 or ATG7 and determine which two best silences the protein of interest. Following this, we will stably silence ATG5 and ATG7 in a panel of PDAC cell lines and assess whether ATG-depleted cells are more sensitive to ERK inhibition.

IV. METHODS

Cell Lines

PDAC cell lines HPAC, PANC-1 were obtained from American Type Culture Collection (ATCC) and lines derived from human PDAC patient xenografts (Pa01C, Pa14C) (Jones et al., 2008) were provided by A. Maitra (MD Anderson, Houston, TX). All lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS and penicillin/streptomycin.

Constructs

The shRNA constructs used were obtained from the UNC-Chapel Hill Lenti-shRNA Core Facility. We obtained five ATG 5 constructs: TRCN0000099432 (ATG5-1), TRCN0000151963 (ATG5-2), TRCN0000150645 (ATG5-3), TRCN0000151474 (ATG5-4), and TRCN0000099431 (ATG5-5); and five ATG7 constructs: TRCN0000007584 (ATG7-1), TRCN0000007587 (ATG7-2), TRCN0000007586 (ATG7-3), TRCN0000007585 (ATG7-4), TRCN0000007588 (ATG7-5).

Lentiviral Transduction

HPAC, PANC-1, Pa01C, and Pa14C cells were plated at a density such that they would be 50-80% confluent in 24 hours. Cells were infected for 8 hours at 37˚C in DMEM containing 8μg/mL polybrene with 500μL of each respective lentivirus. Following infection, cells were allowed to recover overnight in complete media, and then selected with puromycin. The HPAC cells were selected with 1.5μg/mL, PANC-1 cells with 5μg/mL, Pa14C cells with 3μg/mL, and Pa01C cells with 3μg/mL puromycin. Following

selection, cells were maintained in media containing 0.5μg/mL puromycin. Transduction was confirmed via western blotting.

Proliferation Assays

For experiments involving ATG KD cells, parental, ATG5 and ATG7 knockdown cells were plated in black, 96-well plates. Cell plating densities were the following: HPAC: 1,300 cells/well, PANC-1: 2,000 cells/well, Pa14C: 2,500 cells/well, and Pa01C: 2,500 cells/well. Following 24 hours, the cells were treated with SCH772984, over a range of concentrations (19.5nM to 10μM). DMSO was held constant at at 5nM. Cells were incubated at 37˚C for 72hr and proliferation was quantified using a Minimax 300 Imaging Cytometer (Molecular Devices). For cell counting, cells were labeled with either SYTO 61 red fluorescent nucleic acid or Calcein, AM (Invitrogen). Cells were blotted to confirm ATG knockdown.

For experiments involving autophagy inhibitors, parental cell lines were seeded at the following densities: HPAC: 1,500 cells/well, PANC-1: 2,000 cells/well, Pa14C: 2,000 cells/well, and Pa01C: 2,000 cells/well. The next day cells were treated with SCH772984, over a range of concentrations (19.5nM to 10μM) either alone, or in combination with a constant amount of Spautin-1 (Vps34; 1.25μM), SBI-0206965 (ULK1; 2μM), and MRT68921 (ULK1/2; 500nM) (Xcessbio). Cells were labeled and counted as described above.

Western Blotting

Cells were washed in PBS, incubated in lysis buffer (25 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1% (v/v) Triton 100, Phosphatase Inhibitor Cocktails I/II (Millipore), and complete protease inhibitor cocktail (Roche)), and supernatants were retained following microcentrifuge centrifugation. Protein concentrations of whole cell lysates were determined using the Bio-Rad protein assay. Lysates (25-35 μg total protein/lane) were resolved by SDS/PAGE, and the proteins were transferred to PVDF membranes. The filters were blocked in 10% BSA diluted in 20 mM Tris, 135 mM NaCl, and 0.02% Tween 20 and then incubated with the indicated primary antibodies diluted in the same buffer. The primary antibodies were detected with HRP-conjugated secondary antibodies followed by exposure to ECL reagent (GE Healthcare).

V. RESULTS

Autophagy inhibition via inhibition of ULK 1/2 and VPS34 reduces pancreatic cancer cell proliferation

Our lab and others have previously demonstrated that inhibition of autophagy with chloroquine, an inhibitor of lysosomal acidification, reduces PDAC cell proliferation (S. Yang et al., 2011). In order to determine if the effects of chloroquine were due to the inhibition of autophagy and not the general effects of lysosomal inhibition, we decided to study the effects of more specific autophagy inhibitors on PDAC cell proliferation. In order to determine the appropriate concentration to drug cells in combination experiments, dose responses were completed for each autophagy inhibitor. We chose three different autophagy inhibitors, SBI-0206965 (SBI), MRT68921 (MRT), and Spautin-1. SBI targets ULK1, and MRT targets ULK1/2, which is involved in the preinitiation complex of autophagy (Egan et al., 2015; Petherick et al., 2015). This process is activated when the cell is deprived of energy, through the activation of AMPK, a key energy sensor in the cell activated during energy deprivation (Kim, Kundu, Viollet, & Guan, 2011). Spautin-1 inhibits VPS34 which is involved in the formation of the autophagosome initiation complex (Mancias & Kimmelman, 2011). The initiation complex promotes the growth of a membrane that will eventually become the autophagosomal membrane and envelop the macromolecules that will be recycled. We determined the GI50 in four different cell lines: HPAC, PANC-1, Pa14C, and Pa04C (Table 1, Figure 2). We used this information to choose the constant concentration of each autophagy inhibitor to be used in our subsequent ERK inhibitor combination experiments.

Table 1. The GI50s for Spautin-1, SBI-0206965, and MRT68921 in HPAC, PANC-1, and Pa14C cell lines.

Drug	Cell Line	GI_{50}
Spautin-1 (VPS34)	HPAC	16.9 μM
	PANC-1	23.2 μM
	Pa14C	68.5 μM
SBI-0206965 (ULK1)	HPAC	2.3 μM
	PANC-1	1.5 μM
	Pa14C	2.1 μM
MRT68921 (ULK1/2)	HPAC	1.0 μM
	PANC-1	547 nM
	Pa14C	600 nM

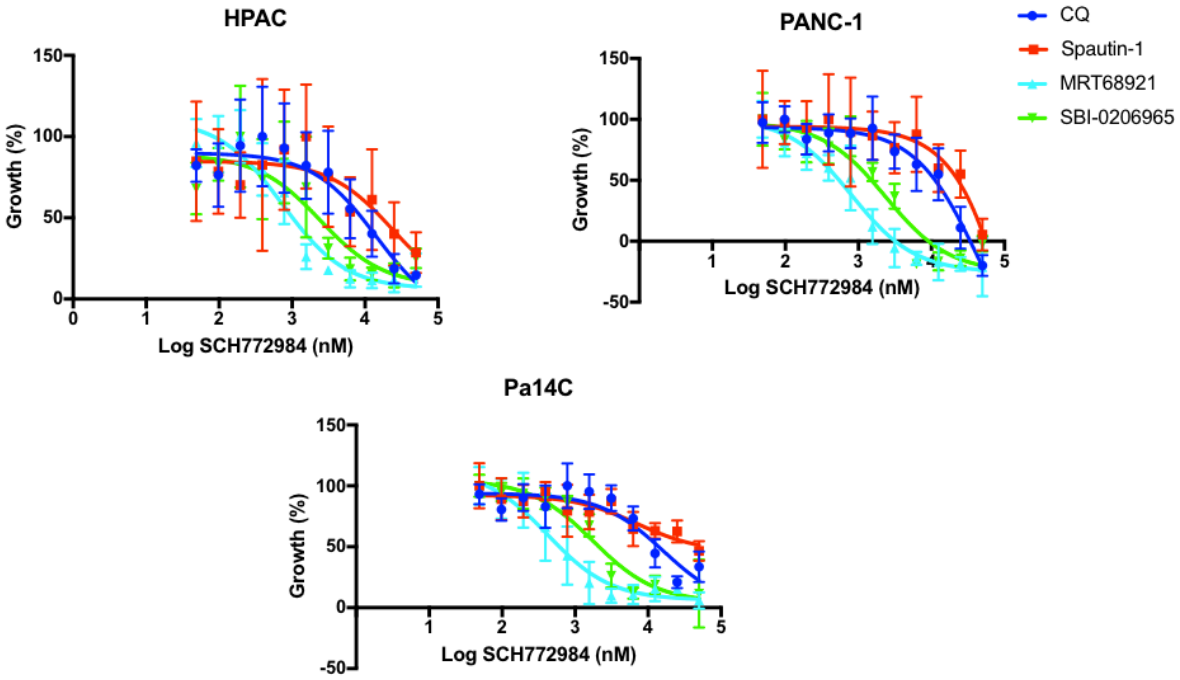

Figure 2. Autophagy inhibition impairs PDAC cell line proliferation. Proliferation of HPAC, PANC-1, and Pa14C (14) cell lines was assayed following 72hr of treatment with a range of the following autophagy inhibitors: Spautin-1, SBI-0206965, and MRT68921, or chloroquine.

Inhibition of ULK 1/2 and VPS34 synergizes with ERK inhibition to further reduce pancreatic cancer cell proliferation

Our laboratory has observed that chloroquine treatment synergized with ERK inhibition to reduce PDAC cell proliferation (Bryant, KL). Chloroquine is routinely used in the clinic for the treatment of malaria and other rheumatological disorders (Rainsford, Parke, Clifford-Rashotte, & Kean, 2015), and other groups have calculated the concentration that is attained in the patient to be on the order of 5-8μM (Alec Kimmelman, NYU, personal communication). Thus, in our lab's previous experiments, chloroquine was held constant at a concentration near this range, while the concentration of ERK inhibitor was varied. In order to compare these studies to what was observed with chloroquine, we held each autophagy inhibitor constant and varied the ERK inhibitor concentration over a range of 19.5nM to 10μM. We used a concentration of 1.25μM for Spautin-1, 2μM for SBI, and 500nM for MRT. Proliferation was quantified following 72hr or inhibitor treatment. Figure 3 illustrates a shift in the growth curve of pancreatic cancer cells when SCH is treated in combination with the autophagy inhibitors.

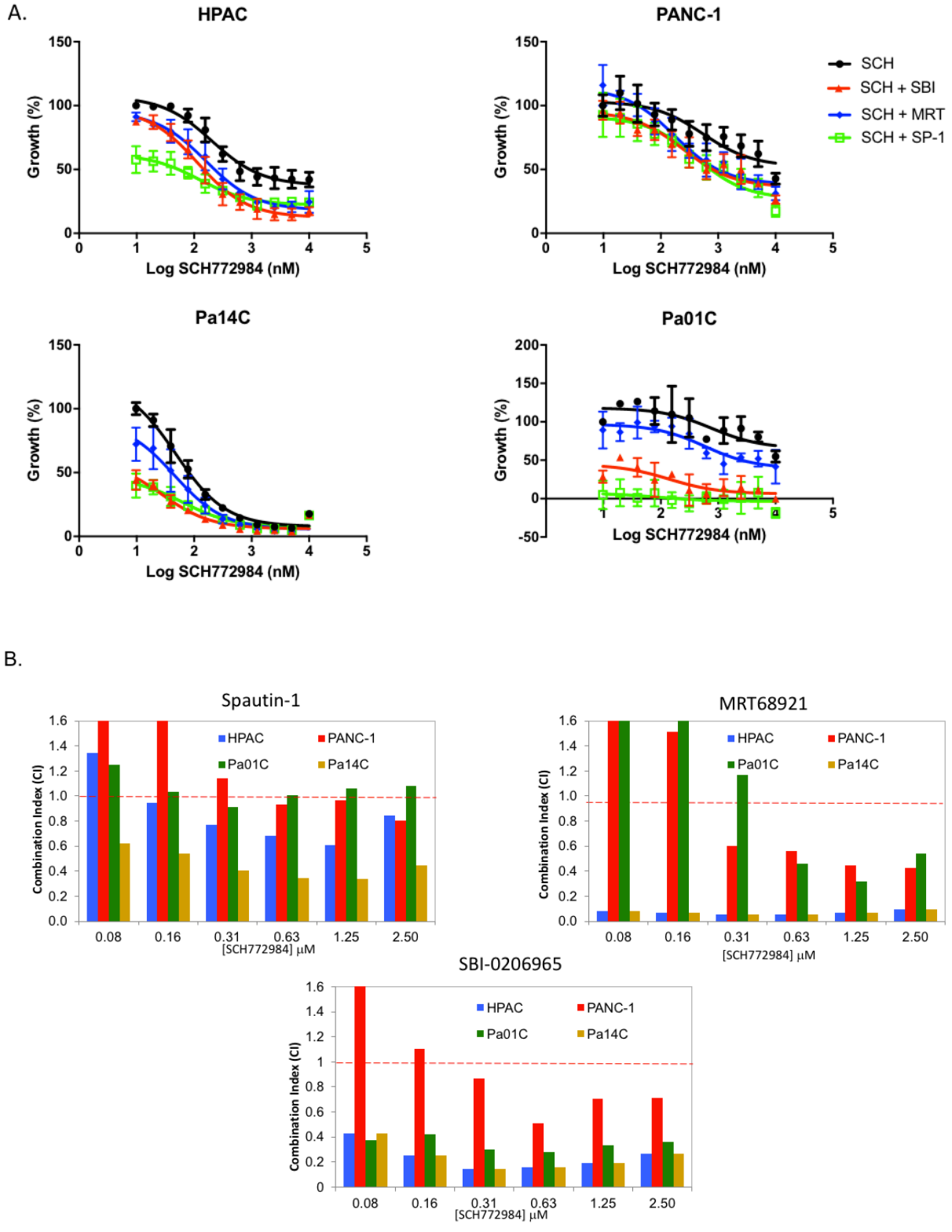

Figure 3. Autophagy inhibitors Spautin-1, SBI-0206965, and MRT6891 synergize with SCH. (A) Proliferation was assayed following 72hr of treatment with a range of SCH concentrations (0.078 to 10mM) and a constant

concentration of each autophagy inhibitor: (List) The combination was assayed in HPAC, PANC-1, Pa14C, and Pa01C cell lines. Data shown is representative of three replicates. (B) CI values were calculated from data displayed in (A). A CI value of <1 (red line) is indicative of synergy.

The combination index (CI) is a measure of synergy between two drugs, with a score of less than one being considered synergistic, and a score of more than one as antagonistic. Clear synergy is observed between either SBI or MRT and SCH. The combination of SCH and MRT consistently showed greater synergy, with lower CIs in each cell line. Spautin-1 synergized best in each cell line when the concentration of SCH was 0.3125 μM or 0.625μM, with the CIs increasing as concentrations decreased or increased (Figure 3). The combination of Spautin-1 and SCH was the least synergistic in the HPAC cell line, and Pa01C cell line. This could be due to the effects of Spautin-1 being significant alone, so the room for improved response to the combination treatment is reduced.

Genetic silencing of ATG5 or ATG7 sensitizes pancreatic cancer cells to ERK inhibition

To complement our inhibitor studies we also employed a genetic approach to silence key regulators of autophagy and determine if this sensitizes cell to ERK inhibition. We inhibited autophagy using shRNA constructs to silence ATG5 and ATG7. ATG5 and 7 are essential for the elongation of the autophagosome membrane, and for the complete formation of the autophagic vesicle (Rabinowitz & White, 2010). We hypothesized that ATG knockdown would phenocopy what we observed with pharmacological autophagy inhibition. Before beginning drug studies, we determined which shRNA constructs could most effectively target ATG5 and ATG7. Cells were transduced with different ATG5 and ATG7 shRNA constructs to determine which gave

the best knockdown. The ATG5 constructs that provided the best knockdown were 2 and 4 (Figure 4A). The ATG7 constructs that provided the best knockdown appear to vary with the cell line they were tested in; therefore, we proceeded with constructs 1, 2 and 3 (Figure 5A).

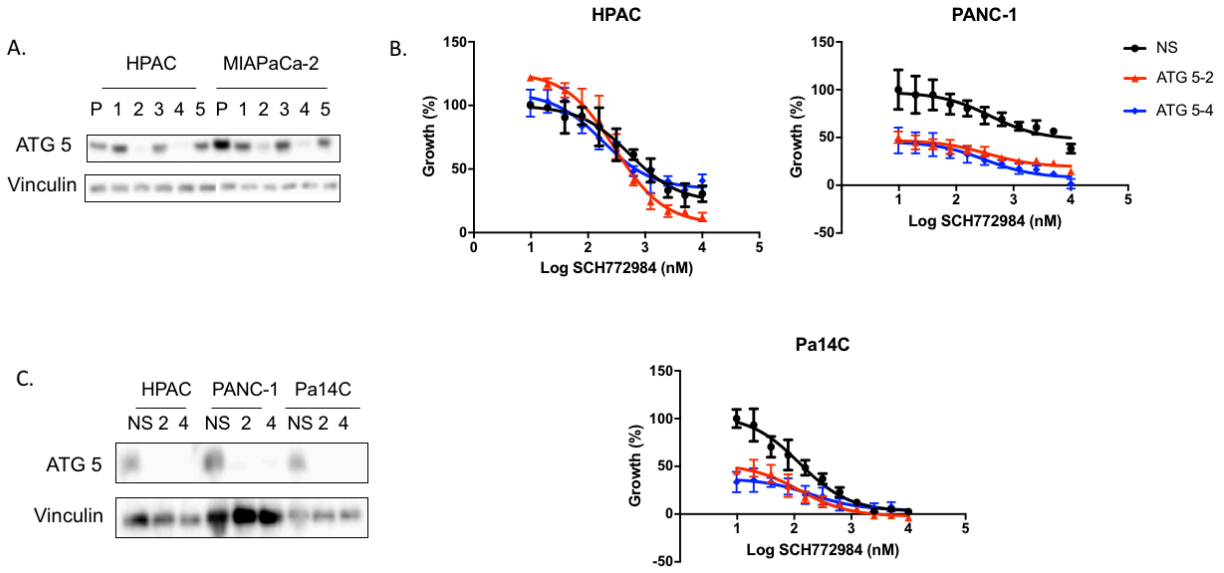

Figure 4. ATG5 KD sensitizes cells to ERK inhibition. (A) ATG 5 was knocked down in HPAC and MIA PaCa-2 cell lines to determine which constructs provided the best knock down, constructs 2 and 4 were chosen for future experiments.(B) Proliferation was assayed following 72hr of ERK inhibition in the HPAC, PANC-1, and Pa14C cell lines. (C) Western blot confirms ATG5 KD.

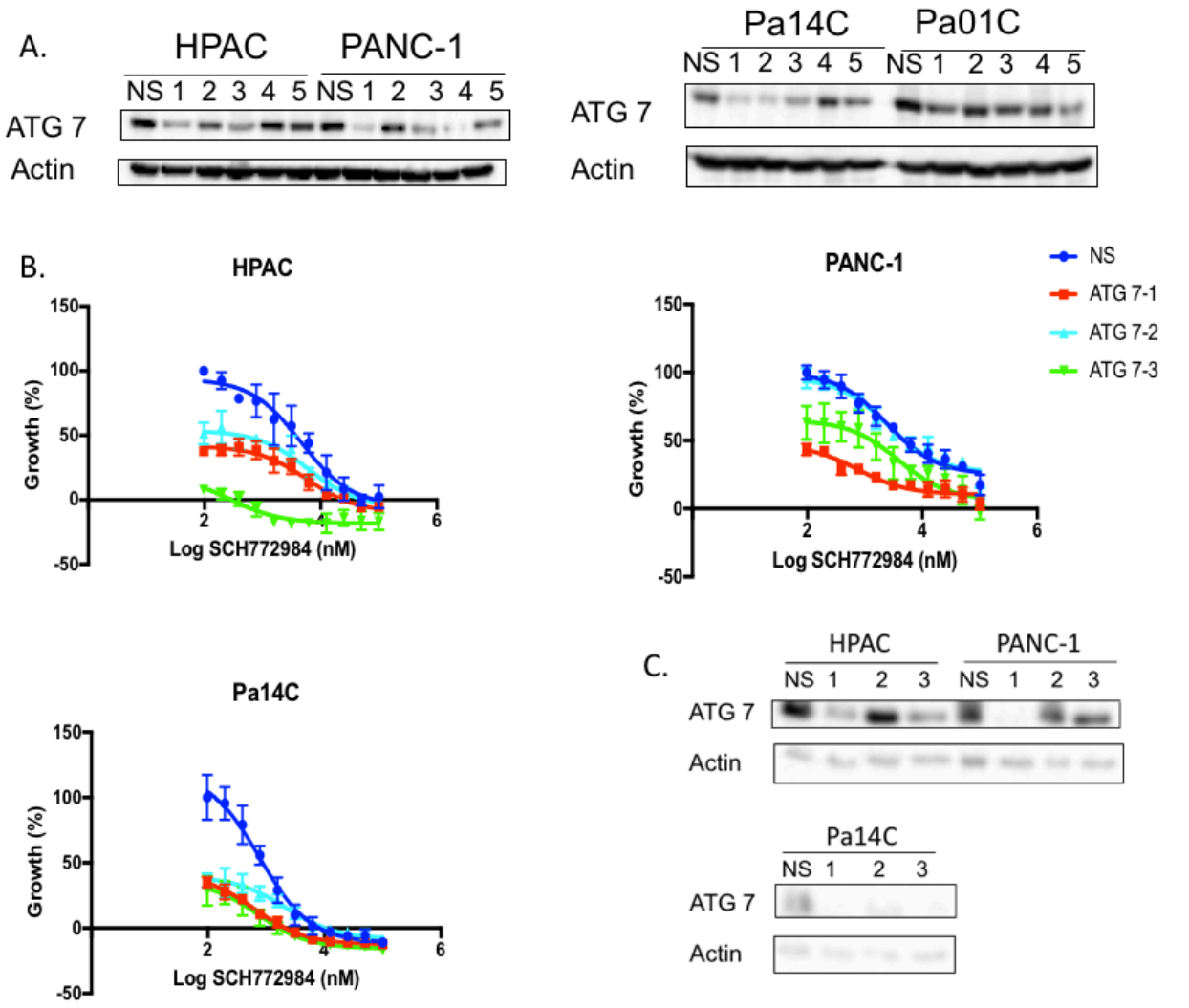

Figure 5. ATG7 KD sensitizes cells to ERK inhibition. (A) ATG 7 was knocked down in HPAC, PANC-1, Pa14C, and Pa01C cell lines to determine which constructs provided the best knock down, constructs 1, 2 and 3 were chosen for future experiments.(B) Proliferation was assayed following 72hr of ERK inhibition in the HPAC, PANC-1, Pa14C, and Pa01C cell lines. (C) Western blot confirms ATG7 KD.

Cells in which ATG5 or ATG7 was genetically silenced were then treated with SCH, and the growth curves were compared to those from cells transduced with a non-targeting shRNA. These experiments were performed in three different PDAC cell lines: HPAC, PANC-1, and Pa14C. The cells were drugged over a range of SCH concentrations (19.5nm to 10μM). The data suggests that cells in which ATG5 has been silence are more sensitive to ERK inhibition than control lines (Figure 4B). This is

evidenced by the fact that the growth curves were shifted to the left in cells with ATG5 knockdown, and suggests that cells incapable of autophagy are more sensitive to ERK inhibition. The knockdown was confirmed by western blotting for ATG5 (Figure 4C). Likewise, the drug studies with ATG7 knockdown also show that cells are more sensitive to ERK inhibition when autophagy is inhibited through genetic silencing of ATG7 (Figure 5B-C).

VI. DISCUSSION

Despite more than three decades of effort, no clinically effective therapies have been developed for *RAS*-mutant cancers. Clearly, new ideas and directions are needed to advance this field. Our lab recently reported that ~50% of RAS-mutant human PDAC cell lines are sensitive to ERK inhibition (Hayes et al., 2016). However, clinical results with RAF and MEK inhibitors emphasize that the RAF-MEK-ERK cascade is not a linear pathway and that compensatory mechanisms abound (Samatar & Poulikakos, 2014). A chemical library screen to identify compounds that enhance ERK inhibitor efficacy, showed that chloroquine, an inhibitor of autophagy, synergizes with ERK inhibition. Ongoing experiments in the lab have demonstrated that ERK inhibition increases autophagic flux. Thus, we hypothesize that autophagy is evoked as a compensatory energy scavenging pathway, in response to the loss of ERK-regulated metabolic processes. These studies were aimed at determining whether the synergy between ERK inhibition and chloroquine is due to autophagy inhibition, the more general effects of chloroquine's inhibition of lysosomal acidification. We show that more specific inhibitors of autophagy synergize with ERK inhibition to levels similar to that of chloroquine. Furthermore, we demonstrate that genetic silencing of ATG5 and ATG7, two important regulators of autophagy, sensitize PDAC cell lines to ERK inhibition. Thus, we conclude that the effects of chloroquine are due, at least in part, to the inhibition of autophagy.

Autophagy inhibitors synergize with SCH in pancreatic cancer cell lines

To determine if the effects of chloroquine were due to autophagy inhibition, we began by using more specific autophagy inhibitors in combination with SCH. Chloroquine was Dr. Bryant's focus primarily due to it has already been approved for use in humans. There are few drugs that target proteins involved in autophagosome formation, as opposed to the lysosome. The CI values for SBI, MRT, and Spautin-1 in combination with SCH are less than one over a range of concentrations. Because a CI value of less than one is indicative of synergy, these CI values and the corresponding growth curves suggest that each shows synergistic effects when used in combination with ERK inhibition. The Pa14C cell line consistently shows higher CI values that even indicate antagonism when MRT is combined with SCH at concentrations greater than 2.5μM and when SBI is combined with SCH at concentrations greater than 5μM. This effect could be because the ULK inhibitors alone have a significant effect on the growth of the Pa14C cell line, thus, because so many cells are already dead, the effect of SCH in combination is not significant enough for it to be considered synergistic. Because these autophagy inhibitors behave very similarly to chloroquine in terms of synergy with an ERK inhibitor, we believe the synergy of chloroquine with SCH is at least partially due to the inhibition of autophagy. There is still clearly a need for well-validated inhibitors that target proteins specifically involved in autophagy, since lysosomal targets also inhibit other pathways.

ATG5 and ATG7 knockdown enhances ERK inhibition in pancreatic cancer cell lines

To complement our drug studies, we also performed shRNA-mediated knockdowns of ATG5 and ATG 7, two essential genes for autophagy, to determine if cells in which autophagy is impaired are sensitized to ERK inhibition. Cells in which ATG5 had been knocked down were more sensitive to SCH treatment than parental cell lines. Growth curves and GI50's showed that construct 4 made HPAC, PANC-1 and Pa14C cells most sensitive to ERK inhibition. A constant problem we encountered during our studies is that we had a low number of cells survive selection after ATG7-3 knockdown. This was particularly a problem in Pa01C cell line. We predict that this could either be due to the virus not entering the cells, or that this particular construct has off-target effects that are lethal to the cell. This resulted in unreliable data from Pa01C cell line due to their low seeding density and high death rate. Although shRNA is considered a precise gene silencing mechanism, there are still off-target effects that should be recognized. The shRNA could include partial sequence complementarity of non-target mRNAs, which can result in effects not attributable to the gene of interest.

Future Directions

Although we have collected data suggesting that the synergy between chloroquine and SCH is at least partially due to the inhibition of autophagy, further studies will determine if macropinocytosis is a factor in the effects seen in the combination treatment. Since macropinosomes are also broken down in the lysosome, chloroquine inhibits it as well and it could help deplete the cancer cell's energy supply.

We would also explore other metabolic processes to determine how the cell rewires after *KRAS* knockdown, and treatment with SCH. Some processes of interest are glycolysis, oxidative phosphorylation and glutamine metabolism.

The data in our studies suggest that the effects seen in the combination therapy of chloroquine and SCH are at least partially due to autophagy inhibition. Further studies could assess these drug treatments in three dimensional organoid cultures, and mouse studies to more accurately represent the effects of the treatment in humans.

I. REFERENCES

Bryant, K. L., Mancias, J. D., Kimmelman, A. C., & Der, C. J. (2014). KRAS: feeding pancreatic cancer proliferation. *Trends in Biochemical Sciences*, *39*(2), 91–100. http://doi.org/10.1016/j.tibs.2013.12.004

Cox, A. D., Fesik, S. W., Kimmelman, A. C., Luo, J., & Der, C. J. (2014). Drugging the undruggable RAS: Mission possible? *Nature Reviews. Drug Discovery*, *13*(11), 828–851. http://doi.org/10.1038/nrd4389

Egan, D. F., Chun, M. G. H., Vamos, M., Zou, H., Rong, J., Miller, C. J., et al. (2015). Small Molecule Inhibition of the Autophagy Kinase ULK1 and Identification of ULK1 Substrates. *Molecular Cell*, *59*(2), 285–297. http://doi.org/10.1016/j.molcel.2015.05.031

Flaherty, K. T., Infante, J. R., Daud, A., Gonzalez, R., Kefford, R. F., Sosman, J., et al. (2012). Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. *The New England Journal of Medicine*, *367*(18), 1694–1703. http://doi.org/10.1056/NEJMoa1210093

Guo, J. Y., Chen, H.-Y., Mathew, R., Fan, J., Strohecker, A. M., Karsli-Uzunbas, G., et al. (2011). Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis. *Genes & Development*, *25*(5), 460–470. http://doi.org/10.1101/gad.2016311

Hatzivassiliou, G., Song, K., Yen, I., Brandhuber, B. J., Anderson, D. J., Alvarado, R., et al. (2010). RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. *Nature*, *464*(7287), 431–435. http://doi.org/10.1038/nature08833

Hayes, T. K., Neel, N. F., Hu, C., Gautam, P., Chenard, M., Long, B., et al. (2016). Long-Term ERK Inhibition in KRAS-Mutant Pancreatic Cancer Is Associated with MYC Degradation and Senescence-like Growth Suppression. *Cancer Cell*, *29*(1), 75–89. http://doi.org/10.1016/j.ccell.2015.11.011

Heidorn, S. J., Milagre, C., Whittaker, S., Nourry, A., Niculescu-Duvas, I., Dhomen, N., et al. (2010). Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF. *Cell*, *140*(2), 209–221. http://doi.org/10.1016/j.cell.2009.12.040

Hsin, I.-L., Sheu, G.-T., Jan, M.-S., Sun, H.-L., Wu, T.-C., Chiu, L.-Y., et al. (2012). Inhibition of lysosome degradation on autophagosome formation and responses to GMI, an immunomodulatory protein from Ganoderma microsporum. *British Journal of Pharmacology*, *167*(6), 1287–1300. http://doi.org/10.1111/j.1476-5381.2012.02073.x

Jones, S., Zhang, X., Parsons, D. W., Lin, J. C.-H., Leary, R. J., Angenendt, P., et al. (2008). Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science (New York, N.Y.)*, *321*(5897), 1801–1806. http://doi.org/10.1126/science.1164368

Kim, J., Kundu, M., Viollet, B., & Guan, K.-L. (2011). AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nature Cell Biology*, *13*(2), 132–141. http://doi.org/10.1038/ncb2152

Mancias, J. D., & Kimmelman, A. C. (2011). Targeting autophagy addiction in cancer. *Oncotarget*, *2*(12), 1302–1306. http://doi.org/10.18632/oncotarget.384

Morris, E. J., Jha, S., Restaino, C. R., Dayananth, P., Zhu, H., Cooper, A., et al. (2013).

Discovery of a novel ERK inhibitor with activity in models of acquired resistance to BRAF and MEK inhibitors. *Cancer Discovery*, *3*(7), 742–750. http://doi.org/10.1158/2159-8290.CD-13-0070

Papke, B., & Der, C. J. (2017). Drugging RAS: Know the enemy. *Science (New York, N.Y.)*, *355*(6330), 1158–1163. http://doi.org/10.1126/science.aam7622

Petherick, K. J., Conway, O. J. L., Mpamhanga, C., Osborne, S. A., Kamal, A., Saxty, B., & Ganley, I. G. (2015). Pharmacological inhibition of ULK1 kinase blocks mammalian target of rapamycin (mTOR)-dependent autophagy. *The Journal of Biological Chemistry*, *290*(48), 28726–28726. http://doi.org/10.1074/jbc.A114.627778

Rabinowitz, J. D., & White, E. (2010). Autophagy and metabolism. *Science (New York, N.Y.)*, *330*(6009), 1344–1348. http://doi.org/10.1126/science.1193497

Rahib, L., Smith, B. D., Aizenberg, R., Rosenzweig, A. B., Fleshman, J. M., & Matrisian, L. M. (2014). Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. *Cancer Research*, *74*(11), 2913–2921. http://doi.org/10.1158/0008-5472.CAN-14-0155

Rainsford, K. D., Parke, A. L., Clifford-Rashotte, M., & Kean, W. F. (2015). Therapy and pharmacological properties of hydroxychloroquine and chloroquine in treatment of systemic lupus erythematosus, rheumatoid arthritis and related diseases. *Inflammopharmacology*, *23*(5), 231–269. http://doi.org/10.1007/s10787-015-0239-y

Rosenfeldt, M. T., O'Prey, J., Morton, J. P., Nixon, C., MacKay, G., Mrowinska, A., et al. (2013). p53 status determines the role of autophagy in pancreatic tumour development. *Nature*, *504*(7479), 296–300. http://doi.org/10.1038/nature12865

Ryan, M. B., Der, C. J., Wang-Gillam, A., & Cox, A. D. (2015). Targeting RAS-mutant cancers: is ERK the key? *Trends in Cancer*, *1*(3), 183–198. http://doi.org/10.1016/j.trecan.2015.10.001

Samatar, A. A., & Poulikakos, P. I. (2014). Targeting RAS-ERK signalling in cancer: promises and challenges. *Nature Reviews. Drug Discovery*, *13*(12), 928–942. http://doi.org/10.1038/nrd4281

Scheffzek, K., Ahmadian, M. R., Kabsch, W., Wiesmüller, L., Lautwein, A., Schmitz, F., & Wittinghofer, A. (1997). The Ras-RasGAP complex: structural basis for GTPase activation and its loss in oncogenic Ras mutants. *Science (New York, N.Y.)*, *277*(5324), 333–338.

Scheidig, A. J., Burmester, C., & Goody, R. S. (1999). The pre-hydrolysis state of p21(ras) in complex with GTP: new insights into the role of water molecules in the GTP hydrolysis reaction of ras-like proteins. *Structure (London, England : 1993)*, *7*(11), 1311–1324.

Siegel, R. L., Miller, K. D., & Jemal, A. (2017). Cancer Statistics, 2017. *CA: a Cancer Journal for Clinicians*, *67*(1), 7–30. http://doi.org/10.3322/caac.21387

Tanaka, T., & Rabbitts, T. H. (2010). Interfering with RAS-effector protein interactions prevent RAS-dependent tumour initiation and causes stop-start control of cancer growth. *Oncogene*, *29*(45), 6064–6070. http://doi.org/10.1038/onc.2010.346

Wolpin, B. M., Rubinson, D. A., Wang, X., Chan, J. A., Cleary, J. M., Enzinger, P. C., et al. (2014). Phase II and pharmacodynamic study of autophagy inhibition using hydroxychloroquine in patients with metastatic pancreatic adenocarcinoma. *The Oncologist*, *19*(6), 637–638. http://doi.org/10.1634/theoncologist.2014-0086

Yang, A., Rajeshkumar, N. V., Wang, X., Yabuuchi, S., Alexander, B. M., Chu, G. C., et al. (2014). Autophagy is critical for pancreatic tumor growth and progression in tumors with p53 alterations. *Cancer Discovery*, *4*(8), 905–913. http://doi.org/10.1158/2159-8290.CD-14-0362

Yang, S., Wang, X., Contino, G., Liesa, M., Sahin, E., Ying, H., et al. (2011). Pancreatic cancers require autophagy for tumor growth. *Genes & Development*, *25*(7), 717–729. http://doi.org/10.1101/gad.2016111