Exploiting Cancer Cell Signaling and Metabolism: Implications for Therapeutic Approach

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A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy,

The City University of New York

2015
The manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

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By Suman Mukhopadhyay

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Over the last decade, metabolic dysregulation in cancer cells has stimulated a significant amount of interest in basic research. It has been established that cancer cells increase glucose uptake and alter the fate of glycolytic and tricarboxylic acid (TCA) cycle intermediates for the synthesis of biological molecules to accommodate high rates of cellular growth and proliferation. Moreover, it is more prominent that some metabolic dysregulations are specific to particular oncogenes. Exploiting the dysregulated metabolic dependency of cancer cells with therapeutic means could represent a novel approach for clinical aspect.

To meet the need of increased anabolic metabolism cancer cells engage in significant induction in uptake of glutamine, conditionally essential amino acid along with glucose. We investigated the impact of glutamine deprivation on cancer cell cycle progression and report here that K-Ras driven cancer cells override a glutamine mediated G1 cell cycle checkpoint and arrest in S-phase of cell cycle. Moreover this differential sensitivity to glutamine in K-Ras mutant cancer cells can be exploited using phase specific cytotoxic drugs. We also show that interfering
with anaplerotic utilization of glutamine sensitizes K-Ras driven cancer cells to the cytotoxic effects of cell cycle phase specific drugs. This study provides the rational for targeting metabolic deregulations in cancer cells.

Next we investigated the connection between the AMP activated protein kinase (AMPK) and phospholipase D (PLD). AMPK, cellular energy sensor, is dysregulated in most cancers whereas PLD is elevated in many cancers. PLD generated phosphatidic acid (PA) is a central metabolite of lipid biosynthesis and regulator of mTOR (mammalian/mechanistic target of rapamycin) signaling node. Although negative impact of AMPK on mTOR has been reported previously little is known about the impact of mTOR on AMPK signaling. We have found that AMPK negatively regulates PLD activity in human cancer cells and in doing so it also suppresses the production of PLD generated PA, which positively regulates mTOR. We also show that PLD and PA suppresses AMPK in an mTOR dependent manner. This study suggests a negative feedback mechanism involving AMPK and PLD/mTOR signals in cancer cells.

To continue our study, we checked the effect of AMPK activator, AICAR (5-Aminimidazole-4-Carboxamide-1-β-4Ribofuranoside) combined with mTORC1 inhibitor rapamycin on cell cycle progression. Rapamycin induces apoptosis in human cancer cells but in higher doses, which are not tolerable in the clinic. We report here that in presence of AICAR, rapamycin inhibits mTOR and induces apoptosis at clinically tolerable doses. We have demonstrated here that by
inhibiting PLD activity, AICAR suppresses the production of PLD generated PA, which interacts with mTOR in a competitive manner with rapamycin. The reduced level of PA sensitizes mTORC2 complexes to rapamycin in tolerable doses leading to suppressed Akt-dependent survival signals and causes apoptosis. This study provides the evidence that tolerable doses of rapamycin in combination with AICAR induces apoptosis in human cancer cells which could be a viable therapeutic option in the clinical.
Acknowledgements

I take this opportunity to express my sincerest thanks and gratitude to my mentor, Dr. David A. Foster, for his excellent guidance throughout my graduate research. I joined his laboratory with no experience in cancer biology but he believed in me and he graciously accepted me as a graduate student. He kept faith on me and on my projects all the way. He taught me the importance of positive attitude for everything. His one-liners “What does this mean” and “Say, what you want to say” I will never forget. He also trained me to become a good thinker rather a good lab technician. My experience in his lab was tremendous and allowed me to not only grow as a good scientist, but also a leader and mentor.

I also extend my deepest gratitude to my committee members Dr. Derrick Brazill and Dr. Patricia Rockwell for providing me valuable suggestions and expert guidance. I am grateful for their patience which allowed me to develop clear understanding of my research. I am honored to have Dr. John Blenis and Dr. Ming Li to serve on my committee and grateful for their time and feedback on my work. I sincerely appreciate their expert opinions and valuable time for my work.

I don’t believe that my work could have been done without support of my lab mates. A big thanks to past and present lab members: Drs. Limei Xu, Paige Yellen, Mahesh Saqcena, Darin Salloum, Amrita Chatterjee and Deepak Menon, Deven Patel, Matthew Utter, Elyssa Bernfeld, Maria A. Frias, Limor Goren and Vishaldeep Vaghela. I was also lucky to have
pleasure of working with two extraordinary undergraduate students, Carol Hosny and Diane Kogan, who contributed tremendously too many aspects of my work.

I thank Inna Rakhlin and Brian Herbert for their support in teaching classes. My work could not have been done without any support from administrators of Biology Department. I want to thank all of my professors, teachers, and friends for their continuous support.

Finally, I thank my family for their continuous support throughout my career. I am happy for this opportunity to publicly acknowledge my family. My mother, who is an amazing and inspiring person and has always had confidence on me and actively encouraged my diverse interests. My mother inspired my dream of being a scientist ever since I was a child. The words of gratitude to my father who has nurtured me with his love and care. Words alone cannot describe the gratitude I owe to my parents Sankar Kumar Mukhopadhyay and Piya Mukhopadhyay. I also thank my beloved wife, Shalini Mukhopadhyay for her support and help in all matters be it small or large. It would not have been possible to reach this stage without her encouragement. I am fortunate enough to have such a supportive and fascinating family members. I owe my family members everything I have done and everything that I am.
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4E-BP1: eIF4E-binding protein 1  
ACC: acetyl-CoA carboxylase  
AICAR: 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside  
AMPK: AMP-activated protein kinase  
eIF4E: eukaryotic initiation factor 4E  
GAP: GTPase activating protein  
LKB1: liver kinase B1  
PA: phosphatidic acid  
mTOR: mammalian/mechanistic target of rapamycin  
PLD: phospholipase D  
Rheb: Ras homolog enriched in brain  
TSC: tuberous sclerosis complex  
ZMP: 5-aminoimidazole-4-carboxamide ribonucleoside monophosphate  
DMEM: Dulbecco’s modified Eagle medium  
FK-BP12: FK506-binding protein 12  
PBS: phosphate buffered saline  
PCNA: proliferating cell nuclear antigen  
PARP: poly-ADP-ribose polymerase  
S6K: S6 kinase  
CDK: cyclin dependent kinase  
EAA: essential amino acids  
GF: growth factors  
R: restriction point  
Rb: retinoblastoma protein
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CHAPTER I

Introduction
1.1 Metabolic reprogramming of cancer cells

The complexity of the cancer has been linked to six basic alterations of cell physiology which together creates malignant cell growth (1). Among them two are related to yielding a phenotype of sustained uncontrolled cell proliferation and escaping from cell death. Mutations in oncogenes and tumor suppressors along with other factors also been shown to influence the malignant cell growth (1). Cancer cells also undergo metabolic reprogramming, which is one of the emerging hallmarks in cancer (1). Under aerobic conditions cancer cells reprogram their glucose metabolism and depend more on “aerobic glycolysis” for their energy production (2). A sustained glycolysis has been linked to enhanced cell proliferation and tumor aggression in vivo (3). Cancer cells undergo significant metabolic reprogramming, which helps to sustain continuous growth and proliferation (4,5). However there is speculation on the involvement of specific oncogenic pathways on this metabolic switch of cancer cells. Altered metabolic rewiring makes cancer cells more proficient for uncontrolled cell growth. Deregulation of several signaling pathways in tumors leads to metabolic alterations in cancer cells (6). Cancer cells evade growth factor dependence by gaining genetic mutations which functionally reprogram signaling pathways to lead uncontrolled cell proliferation. Evidence from recent research suggests that metabolic reprogramming of signaling pathways lead to constitutively activate uptake and metabolism of nutrients, which generates survivals signals and promotes cell growth (2). Recent findings also suggest that several differences are present in cancer cells compared to their normal counterparts. Since metabolism is one of the precursors for cellular growth, better understanding of cancer cell metabolism is required for targeting cancer cells specifically. Differences in metabolism in cancer cells
compared with non-cancer cells would be targets for clinical intervention. Clear understanding of altered metabolism in cancer cells will help to create new targets for therapeutic interventions.

### 1.2 Glutamine metabolism in cancer cells

Over the last decade, there has been a resurgence of interest in metabolism stimulated largely by the observation that there is a metabolic reprogramming of cancer cells (7). To meet the increased anabolic demand to accommodate cell growth and proliferation, cancer cells elevate glucose uptake and reprogram the fate of glycolytic and tri-carboxylic acid (TCA) cycle intermediates towards synthesis of amino acids, nucleotides, and lipids needed for the cell to double its mass and divide. In dividing cells, citric acid, which is synthesized from the condensation reaction between acetyl-CoA and oxaloacetate in the first step of the TCA cycle, exits the mitochondria and regenerates acetyl-CoA, which is then used for the generation of lipids needed for membrane biosynthesis. The exit of citric acid from the mitochondria and the TCA cycle creates a need for anaplerotic replenishment of TCA cycle intermediates downstream of citric acid. The major source for anaplerotic replenishment of TCA cycle intermediates is the "conditionally essential" amino acid glutamine (Gln). It has been shown that Gln is an actively metabolized substrate for cancer cells (8). Although it has been reported that glucose deprivation causes cell death in several cancer cells whereas Gln deprivation arrests cell proliferation (3,9) it is not yet completely clear how glutamine is required for to support uncontrolled cancer cell growth.

Gln is most abundant amino acid in human plasma and is involved in several metabolic pathways. Cancer cells consume more Gln compared to their normal counterparts in
higher rates (4). After being transported into mitochondria Gln is first deaminated to glutamate, and then to α-ketoglutarate via either glutamate dehydrogenase or transamination reactions with α-keto-acids such as oxaloacetate to generate aspartate (Figure 1.1). As much as 25% of Gln gets incorporated into membrane lipids (8) – indicating that a substantial amount of Gln is converted to citric acid for export to the cytosol for fatty acid synthesis. Gln-derived α-ketoglutarate is also critical for redox balance and the generation of NADPH via the conversion of malate to pyruvate (Figure 1). These observations underscore the critical importance of Gln as a nutrient source in dividing and metabolically reprogrammed cancer cells.

Anaplerotic Utilization of Glutamine

![Diagram of anaplerotic glutamine utilization](image)

Figure 1.1. Schematic overview of anaplerotic Gln utilization. Adapted from(10): Gln is first deaminated to glutamate by glutaminase (GLS). Glutamate is then converted to α-ketoglutarate via either glutamate dehydrogenase (GDH) or transamination catalyzed by glutamate oxaloacetate transaminase (GOT), which uses oxaloacetate as the amino group acceptor to generate aspartate.
Aminooxyacetate (AOA) inhibits GOT and therefore suppresses generation of \(\alpha\)-ketoglutarate from Gln-derived glutamate. Aspartate is critical for redox balance and NAPDH production (11) and the generation of citrate for fatty acid synthesis. Gln is also important for redox balance via the conversion of malate to pyruvate, which also generates NADPH.

Consistent with the importance of Gln as a nutrient for dividing cells, we recently identified a late G1 Gln-dependent cell cycle checkpoint that could be distinguished from two other late G1 checkpoints – one that was dependent on essential amino acids, and another that is dependent on mTOR(12). All three metabolic checkpoints were clearly distinguished from the mid-G1 growth factor-dependent Restriction Point (12).

1.3 Mammalian Target of Rapamycin (mTOR)

1.3.1. Overview on mTOR signaling

mTOR, the mechanistic/ mammalian target of rapamycin, is a serine/threonine protein kinase that regulates cell metabolism, growth, proliferation and survival in response to environmental signals provided by growth factors, nutrients, oxygen, energy and stress (13). mTOR acts as the catalytic subunit in two large multiprotein complexes: mTORC1 and mTORC2. The binding partner Raptor defines mTORC1 (14,15), whereas Rictor defines mTORC2 (16,17). Both complexes contain several other structural and regulatory components required for their function (18-23). mTORC1 phosphorylates several downstream targets to promote biosynthesis of proteins, lipids and nucleotides, while turning off autophagy, which is a major macromolecule degradation mechanism (24). S6K and 4E-BP are by far the best characterized mTORC1 targets, since they were the first to be discovered several decades ago (25,26). S6K phosphorylation on threonine
389 (Thr389) (27) leads to ribosome biogenesis. Sequential phosphorylation of 4E-BP at the residues threonine 37/46 (Thr37/46), serine 65 (Ser65) and threonine 70 (Thr70) prevents binding and sequestration of the eukaryotic translation initiation factor eIF4e, which drives CAP-dependent translation initiation (28,29). Much less is known about mTORC2 relative to mTORC1 (30). However, this complex plays seminal functions in cell metabolism and survival through the phosphorylation of serine 473 (Ser473) of AKT, one of the main cellular proto-oncogenes(31). The general overview of mTOR signaling is in fig 1.2.

Mutations in tumor suppressors and/or oncogenes upstream of mTORC1 result in increased activation of mTORC1, making such cancer cells distinct from their normal counterparts and especially vulnerable to mTORC1 inhibition (32). Altered cell metabolism, uncontrolled proliferation and enhanced survival through the loss of apoptotic programing are major hallmarks of cancer (1). Since mTORC1 is a key regulator of such cellular functions it is not surprising that mTORC1 hyper-activation has been found in most human cancers (33). This opens the possibility of selectively targeting cancer cells through inhibition of mTORC1. As its name implies, mTOR is the target of rapamycin, a macrolide antibiotic with critical anti-proliferative properties. Much of what is currently known about mTOR came from studies trying to understand the mode of action of rapamycin (34). Rapamycin first binds to its intracellular receptor, FKBP12, and then binds to and inhibits mTOR (35). Rapamycin does not directly inhibit the mTOR kinase. Instead, it works as an allosteric inhibitor through binding to the FRB (FKBP12-Rapamycin binding) domain of mTOR (amino acid residues 2025 to 2114), which is located immediately N-terminal to the kinase domain (36). Binding of rapamycin causes
conformational changes in mTOR (37) that affect complex assembly (38) and substrate binding (39) mTORC1 is sensitive to rapamycin while mTORC2 is less sensitive to the drug. Within one hour, rapamycin disrupts the association between mTOR and raptor but not with rictor (40). Raptor plays a key role in mTORC1 substrate recognition and thus rapamycin also affects substrate binding (41). However, 24hr or longer exposures to rapamycin also inhibit mTORC2 (42). Unassembled mTOR bound to rapamycin fails to assemble into mTORC2 and turn over the active complex. Thus mTORC2 activity is lost over time.

Figure 1.2. Nutrient signals to mammalian target of rapamycin (mTOR) Adopted from (43): Regulation of mTOR has many inputs. The phosphatidylinositol-3-kinase (PI3K) input involves the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) to PIP3, which recruits and activation (PDK1), which in turn phosphorylates Akt at Thr308. Subsequently, Akt phosphorylates and suppresses the GTPase-activating protein (GAP) activity of the tuberous sclerosis complex (TSC) consisting of TSC1 and TSC2 (TSC1/2). Suppression of TSC1/2 activates the GTPase Rheb, which than
activates mTORC1 via the activation of phospholipase D1 (PLD1) and suppression of FKBP38 whereby elevated PLD activity generates the phosphatidic acid (PA) necessary for the formation of the mTORC1 complex and the dissociation of FKBP38 from mTORC1. This pathway is also modulated by AMP-activated protein kinase (AMPK) which, in combination with the tumor-suppressor LKB1, activates TSC1/2, which then suppresses Rheb and thus mTOR – under conditions where ATP levels are low and AMP levels are high. Akt is also phosphorylated by mTORC2 at Ser473 in response to insulin and insulin-like growth factor-1 (IGF1) in a PLD-dependent manner. Phosphorylation at this site has been correlated with altered substrate specificity and kinase activity for Akt. Insulin also increases the level of glucose transporters and the uptake of glucose. A common theme in this complex signaling network leading to mTORC1 activation is that it is highly sensitive to the presence of glucose and amino acids – nutrients needed for cell growth.

1.3.2. Action of Rapamycin on mTOR signaling

Rapamycin derivatives known collectively as rapalogs are presently FDA-approved drugs for the treatment of certain cancers (44). Results of clinical trials have not met the high expectations for rapamycin in cancer therapy. Intrinsic and acquired resistance to rapamycin have been widely observed both in pre-clinical and clinical studies (34). mTOR inhibition through rapamycin has traditionally been measured through the levels of phosphorylation of Thr389-S6K1 and cell proliferation/viability assays. It has long been known that the tolerated dose of rapamycin, at a low nanomolar range, does not inhibit P-Thr389-S6K1 equally between different cancer cell types (45,46). Some require higher doses of rapamycin. In recent years it has also become evident that the low nanomolar doses of rapamycin required for P-Thr389-S6K1 inhibition are not enough to target phosphorylated 4E-BP1 (47) (48,49). Higher doses of rapamycin are required for
complete inhibition of 4E-BP1(50). Moreover, inhibition of mTORC2 with prolonged nanomolar doses will not happen in some cancer cell types(42), which might require higher doses of rapamycin for inhibition of P-Ser473-Akt.

1.3.3. Differential sensitivity of mTORC1 target P-S6K to rapamycin

Initial evidence that different cancer cells respond differently to rapamycin came from studies with rapamycin or rapalogs with cultured cancer cell lines. More recent studies with mouse tumor xenografts and cancer samples have led to identical conclusions (34). Some cancer cells show extreme sensitivity to inhibition of P-S6K1 with rapamycin, with IC50s between 0.5 and 10 nM. Other cells show good sensitivity to rapamycin with IC50s around 100nM. Overall, cancer cells with increased activation of the PI3K growth factor pathway tend to be sensitive to rapamycin in low nanomolar doses (1-100 nM). These cells have high basal levels of P-Thr389-S6K1 and P-Ser473-Akt and these have been found to be good predictors of rapamycin sensitivity by several independent studies (51). These cells are resistant to rapamycin and they have been the subject of many studies trying to underscore the mechanisms of resistance to rapamycin. Several factors have been associated with intrinsic resistance to rapamycin. The list is long and includes mutations in mTOR and FKBP12, mutations in proteins associated with DNA damage responses, reduced levels of 4E-BP or negative regulators of the cell cycle, among others(52) .Importantly, recent studies have shown that there is a positive correlation between PLD activity and the dose of rapamycin required for P-S6K1 inhibition (53,54).Our lab and others have shown that PLD-generated PA is required for mTOR
activity in response to nutrients (55-58) and that PA interacts with the FRB domain of mTOR in a competitive way with rapamycin (54,59). In breast cancer cells, cell growth inhibition can be achieved at 20 nM concentration of rapamycin for MCF-7 cells whereas 20 µM is required for MDA-MB-231 cells (53). Regarding P-S6K, 0.5 nM rapamycin are enough to suppress phospho-Thr389-S6K in MCF7 cells whereas 20 nM is required in MDA-MB-231 cells. The latter have a 10-fold increase in PLD activity relative to MCF-7 cells. MDA-MB-231 cells produce more PA and therefore require higher amounts of rapamycin to compete off PA and inhibit mTOR. Critically, elevation of PLD activity by 3 fold in MCF-7 cells leads to a 10-fold increase in the dose of rapamycin required to inhibit P-Thr389-S6K1. Similarly, a 3-fold reduction of PLD activity in MDA-MB-231 cells sensitizes these cells to rapamycin by lowering the dose required for S6K1 inhibition from 50nM to 5 nM (10 fold) (53). These findings give PLD-generated PA a key role in mTOR activation in a manner that is competitive with rapamycin. Importantly, they open the possibility of mTOR inhibition in rapamycin resistant cells through combined therapy with PLD inhibitors.

1.3.4. Differential sensitivity to rapamycin by different mTORC1 targets - S6K1 and 4E-BP1

Historically, mTOR inhibition has been probed through the levels of P-Thr389-S6K1. There is a fundamental reason for that: researchers did not feel comfortable with results achieved with 4E-BP1. Several factors were responsible for this uncertainty. 4E-BP1 has 4 mTOR-dependent sites that show different levels of inhibition at the nanomolar range doses. In addition, mammals have three 4E-BP genes and proteins and therefore
there might be antibody cross-reactivity with the same sites in 4E-BP2 and 4E-BP3. Data generated for phospho-4E-BP1 was difficult to interpret and therefore was frequently excluded. It is now widely accepted that low nanomolar doses of rapamycin often lead to complete inhibition of S6K but only partial inhibition of 4E-BP1. Two lines of research, one with mTOR catalytic inhibitors (47-49) and one with high dose rapamycin (50), helped establish the idea of partial inhibition of 4E-BP1 by low dose rapamycin. The development of ATP-competitive kinase inhibitors of mTOR by several independent groups (47-49) showed that these compounds induced a more profound inhibition of mTORC1. They lead to complete suppression of phospho-4E-BP1, which translated into a deep cell cycle arrest and inhibition of proliferation. Since the catalytic inhibitors of mTOR affect both mTORC1 and mTORC2, there was concern that inhibition of mTORC2 contributed to the profound proliferation inhibition. However, identical proliferation inhibition kinetics achieved in WT and rictor -/- (47) or Sin1 -/- (48) MEFS showed that it was indeed due to complete inhibition of phospho-4E-BP1. Importantly, studies using micromolar concentrations - high dose - rapamycin lead to the same conclusions. Data from Yellen et al., 2011 (50) suggests that low dose rapamycin induces a weak dissociation between mTOR and raptor since the presence of a protein cross-linker can recover the interaction. This is enough to prevent S6K phosphorylation but not 4E-BP1 phosphorylation. Significantly, at micromolar doses, rapamycin causes a strong if not complete dissociation between mTOR and raptor, which can no longer be recovered by protein crosslinking. In these conditions there is complete suppression of phospho-4E-BP1. Blenis and colleagues have suggested that S6K interacts with Raptor more weakly that with 4E-BP1 (39,41). This supports the idea that low doses of rapamycin have a weak effect on Raptor
but which are enough to prevent binding of S6K, whereas 4E-BP1 requires a more profound destabilization of mTORC1 to prevent its binding to Raptor. A very important aspect of the study of Yellen et al., (50) is that complete inhibition of phospho-4E-BP1 lead to a complete cell cycle arrest and apoptotic cell death in certain cell growth conditions. Apoptosis was due to complete suppression of eIF4E by 4E-BP1 since it could be reproduced by knocking down eIF4E and it could be rescued by knocking down 4E-BP1. This also cleared any concerns regarding the specificity of high dose rapamycin by showing a specific effect on 4E-BP1. The fact that high dose rapamycin can kill cancer cells suggests that complete inhibition of mTORC1, in certain conditions, can be cytotoxic instead of cytostatic, which would have profound implications in cancer therapy. Additionally, these results might explain the reason why for so many decades researches tried to use rapamycin to cause cancer cell death without much success: nanomolar doses of rapamycin lead to partial inhibition of 4E-BP1 only. Data from the clinic support a critical role for 4E-BP1 in cancer development. An increase in 4E-BP1 phosphorylation status reduces overall survival of metastatic melanoma patients (60). High dose rapamycin can lead to apoptosis, but it cannot be tolerated by cancer patients. Therefore finding a way to achieve complete mTORC1 inhibition at the well tolerated nanomolar doses of rapamycin is a major concern. This could potentially be achieved through the combination of low dose rapamycin with other drugs with an inhibitory effect on mTORC1 such as PLD inhibitors.
1.3.5. **Differential inhibition of mTORC1 and mTORC2 by rapamycin**

The existence of feedback loops downstream of mTORC1 that impact on cell survival pathways can impose limits to the efficacy of rapamycin-based treatments. In response to suppression of S6K phosphorylation, induction of Akt phosphorylation has been reported in some cancer cells (61). Thus mTORC1 inhibition by rapamycin results in feedback activation of mTORC2 and phosphorylation of the survival kinase AKT. This negative feedback loop was responsible for saving cells from apoptosis induced by high dose rapamycin in breast cancer (50) and pancreatic cancer cells (62). Importantly, inhibition of mTORC2 or suppression of AKT phosphorylation restored the apoptotic effect of high dose rapamycin. These results suggest that the treatment of cancer cells that display the negative feedback loop between S6K and AKT might require inhibition of both mTORC1 and mTORC2. mTORC2 is resistant to rapamycin in both low and high dose. Regardless of dose, prolonged treatment with rapamycin can lead to suppression of mTORC2 in some cancer cells (42). This suggests that mTORC2 has a long half-life and that over time rapamycin prevents free mTOR bound to rapamycin from assembling into new mTORC2.

1.3.6. **Advantages of rapamycin for therapeutic purposes**

Rapamycin is an allosteric inhibitor of mTOR that has been widely used in both pre-clinical and clinical settings. A second generation of mTOR inhibitors has more recently been developed. These are ATP-competitive inhibitors that disable the kinase
activity of mTOR. These compounds present two major advantages relative to rapamycin: they present greater efficacy in mTOR inhibition and they inhibit both mTORC1 and mTORC2. However, like other ATP-competitive inhibitors, these compounds seem to be associated with greater toxicity and lower specificity. Another pitfall of the mTOR catalytic inhibitors comes from shorter durations of activity. In contrast, rapamycin is a natural product that allosterically inhibits mTOR with a very high specificity. Even high doses of rapamycin do not seem to produce off-target effects. However, high doses of rapamycin cannot be administered in the clinic and this might be the main reason why nanomolar doses of rapamycin have not lead to more impressive results in clinical trials.

In order to take advantage of low toxicity and high specificity of rapamycin for mTOR, there need to be a means for making rapamycin more efficient at lower doses. One possible strategy is to introduce rapamycin through a vehicle. For example, tagging rapamycin to glucose, which is taken up preferentially by most cancer cells. Such a method could help deliver tolerable levels of rapamycin and cause greater toxicity in the tumor microenvironment.

1.4 Phospholipase D (PLD) and Phosphatidic Acid (PA)

1.4.1 Overview of PLD and PA

Phospholipase D is an enzyme that catalyzes the hydrolysis of phosphatidylcholine into phosphatidic acid (PA) and choline. PLD is evolutionary conserved lipase from bacteria to mammals. Two isoforms of PLD (PLD1 and PLD2) are exist in mammals
among them PLD1 is more responsive to growth factors and nutritional stimuli (55,57,63).

PLD1 is localized to perinuclear regions of the cell with a pattern consistent with that of the Golgi apparatus, endoplasmic reticulum and endosomes (64). It has been reported that PLD1 also localized in the plasma membrane after stimulated by phorbol 12-myristate-13-acetate (PMA), insulin (65,66). On the other hand, most reports demonstrate that PLD2 localizes to the plasma membrane (67), but it has been found in the cytosol, vesicular compartments (68).

PLD has been implicated in a number of cellular activities, including cell migration, exocytosis and receptor endocytosis (63,69). These functions are vital for the cell survival but are also common contributors to tumorigenesis. Not surprisingly, PLD, whose activity is elevated in a large number of human cancers, has been implicated in the survival signals that suppress apoptosis when the cells are subjected to stress of serum withdrawal (70,71). Mammalian PLD proteins are highly responsive to growth factor and hormone stimulation. Numerous reports provide evidence which show that PLD activity can be stimulated by epidermal growth factor (EGF), platelet derived growth factor (PDGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and insulin in various systems. In some of these cases, PLD proteins have been shown to physically associate with the receptors of the aforementioned growth factors. Inhibitors of tyrosine kinases such as genistein inhibited PLD activity stimulated by various agonists. On the other hand, inhibition of protein tyrosine phosphatases with compounds such as vanadate, stimulated PLD activity (72).

PA can function as a second messenger to activate various kinases including mTOR. In addition, PA is an essential substrate for enzymes involved in the synthesis of
glycerophospholipids and triacylglycerols. PA stands in the center of lipid synthesis, both for membrane phospholipids and triglyceride synthesis (43,73).

1.4.2 Role of PA in mTOR signaling

PA is a phospholipid that binds to the FRB domain of mTOR (59) to promote complex assembly and stability (54). Detailed study on how the levels of cellular PA and the levels of activity of its generating enzyme, phospholipase D, can explain required higher doses of rapamycin to achieve full inhibition of mTOR by some cancer cell types.

Recent findings have shown that strong reductions in intracellular levels of PA lead not only to mTORC1 inactivation but also mTORC2 inactivation (54). These findings support the idea that the affinity of PA for mTORC2 is greater than for mTORC1. PA allows a greater stability to mTORC2 compared to mTORC1. Critically, PA binds to mTOR in a competitive manner with rapamycin thus the differential sensitivity of mTORC1 and mTORC2 to rapamycin can be explained by the differential affinity of mTORC1 and mTORC2 to PA (Figure 1.3). The higher sensitivity of mTORC1 to rapamycin is consistent with the lower affinity of PA for mTORC1. Similarly, a higher affinity of PA to mTORC2 is coherent with lower sensitivity to rapamycin. In other words, PA easily dissociates from mTORC1 in the presence of rapamycin but not from mTORC2. The dissociation constant for PA and mTORC2 is therefore much lower than the dissociation constant for PA and mTORC1 (Figure 1.3). A PA-centered model for the differential sensitivity of mTORC1 and mTORC2 to rapamycin, though oversimplified, opens the possibility of new routes of cancer therapy with drugs that significantly lower the levels of PA in combination with rapamycin at tolerable nanomolar doses.
Fig 1.3. Model for the effects of PA on mTORC1 and mTORC2: The rate constant for the dissociation of mTORC1 to PA and mTOR (K_{D1}) is greater than rate constant for the dissociation of mTORC2 to PA and mTOR (K_{D2}). There are less disassociations of PA from mTORC2 compare to mTORC1. The efficient ability of rapamycin-FKB12 to suppress mTORC1 and mTORC2 depends on the availability of mTOR availability to bind with rapamycin-FKB12. Reduction of PA by PLD inhibitors would reduce the concentration of rapamycin-FKB12 needed to bind to and inhibit mTOR.

Our lab has demonstrated that inhibition of PLD activity leads to lower intracellular levels of PA, which in turn allow rapamycin to inhibit both mTORC1 and mTORC2 at lower doses. PLD inhibitors (74) have been developed by the group of Alex Brown and are presently undergoing clinical trials (75) for certain human disorders and have also been reported safe and efficient. The combination of PLD inhibitors with low dose rapamycin has great potential as an anti-cancer therapy and deserves further research to establish major beneficial properties and pitfalls. The schematic representation of this strategy is been proposed in Fig 1.4. Suppressing PLD activity by PLD inhibitors – makes mTORC2 sensitive to rapamycin at clinically tolerated doses. Thus, while the combination of PLD inhibitors and rapamycin might seem redundant – they both suppress mTORC1, and as
a consequence, make mTORC2 responsive to tolerated doses of rapamycin leads to suppression of mTORC2 as well as mTORC1.

![Diagram of mTORC signaling pathways under different conditions](image)

**Fig 1.4:** Schematic representation for the role of PA on efficacy of rapamycin: Low doses of rapamycin treatment inhibit S6K phosphorylation very efficiently but fail to inhibit 4E-BP1 phosphorylation whereas high doses of rapamycin treatment efficiently inhibit both of them but in clinically non tolerable doses. It is arguable that combination of PLD inhibitors and low dose rapamycin treatment would inhibit mTORC1 efficiently. Additionally this duel treatment would inhibit the survival signal generated from mTORC2, which would result in synthetic lethal phenotype in cancer cells.

### 1.5 AMPK

#### 1.5.1 Overview of AMPK

AMPK is a serine-threonine kinase which acts as a ‘energy sensor’ that responds to the changes in cellular metabolic ATP levels(76). Once cellular ATP levels goes down by various stressful conditions such as oxygen deprivation and heat shock, AMPK gets activated and functions by inhibiting energy consuming process while activating ATP-
producing pathways. Thus AMPK works to optimize the total cellular ATP levels in order to maintain critical physiological processes or for survival in response to stress (76,77). The heterotrimeric AMPK contains a catalytic α-subunit and regulatory β and γ subunits. The α-subunit contains critical feature for enzymatic activity, the N-terminal kinase domain contains a threonine residue (Thr-172) whose phosphorylation is essential for AMPK activation (76). Mammalian AMPK is activated by 5'-AMP (AMP), whereas it is inactive unless phosphorylated on its threonine residue (Thr-172) by upstream kinases (78,79). Phosphorylation of AMPK at Thr172 is critical for enzymatic activity and upstream kinase LKB1 is responsible for this phosphorylation. LKB1 activates AMPK through phosphorylation of Thr172 and is essential for activation of AMPK by exercise (80) or widely used drug for diabetes metformin(81). Mammalian cells lacking LKB1 expression, cannot activate AMPK upon treatment of anti-diabetic drugs phenformin or 5-aminoimidazole 4-carboxamide riboside (AICAR) (82).
Fig 1.5: Pathways regulated by activated AMPK (Adopted from (77)): AMPK suppresses the activity of various proteins involved in anabolic pathways (top half of wheel) whereas catabolic pathways are switched on by AMPK.

1.5.2 AMPK signaling network

AMPK acts as an energy sensor of the metabolic state of the cell. AMPK regulates several metabolic pathways as it acts like a metabolic switch of the cellular metabolism. Upon activation AMPK leads to energy preservation for cell survival, inhibits all ATP consuming pathways while it also activates ATP generating signaling pathways for cellular survival. Pathways regulated by activated AMPK is illustrated in figs 1.5, 1.6. After activation, AMPK controls multiple targets including mTOR signaling and lipid biogenesis pathways. AMPK plays a major role in regulating cell growth, proliferation and autophagy through inhibiting mTOR signaling cascade(79). AMPK inhibits a number of key metabolite enzymes that consume ATP but are not immediate essential for survival. These proteins include acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), HMG-CoA reductase, mTOR (77). AICAR is one of the activator of AMPK most widely used in the cell culture studies. AICAR is a precursor of zinc metalloproteinase (ZMP), which competes with AMP for binding to AMPKγ subunit. Activated AMPK by AICAR treatment inhibits amino acid induced activation of p70S6K (83). AMPK phosphorylates and activates tumor suppressor TSC2 which subsequently inhibits mTOR. AMPK also phosphorylates mTORC1 partner raptor and inhibits the mTORC1 complex (84).
Fig 1.6: The LKB1/AMPK/mTOR signaling pathway (Modified from (79)): Activated AMPK upon treatment of AICAR critically inhibits two major anabolic pathways. AMPK phosphorylates and activates TSC2, negative regulator of mTOR pathway. By inhibiting mTOR, AMPK negatively regulates protein translation process whereas AMPK also negatively regulates lipid biogenesis by inhibiting acetyl CoA carboxylase activity.
CHAPTER II

Methods and Materials
2.1 Cells, cell culture conditions

The Calu-1, DU-145, LNCaP, MCF-7, MDA-MB-231, Panc-1 and BJ-hTERT cells used in this study were obtained from the American Tissue Type Culture Collection. LNCaP cells were maintained in RPMI-1640 and all the other cells were maintained in DMEM supplemented with 10% fetal bovine serum (Sigma) and penicillin/streptomycin (Sigma). Transfections were performed using Polyfect (Qiagen) for plasmid transfection and Lipofectamine RNAiMAX (Invitrogen) for siRNA transfection according to manufacturer’s instructions.

2.2 Materials

Reagents were obtained from the following sources: Antibodies against phospho-Akt (T308 and S473), phospho-S6K (T389), phospho-4EBP1 (T37/46), phospho-ERK1/2 (T202/Y204), phospho-Rb (S807/811), cyclin E, cleaved PARP, and actin were obtained from Cell Signaling; antibody against p27 was obtained from Santa Cruz Biotechnology; antibodies against cyclin A, cyclin B, and cyclin D were obtained from BD Biosciences; and anti-mouse and anti-rabbit HRP conjugated secondary antibodies were obtained from Promega. DMEM (D6429), DMEM lacking Gln (D5546), RPMI-1640 (R8758), RPMI-1640 lacking Gln (R0883), dialyzed fetal bovine serum (F0392), and glutamine (G7513), aminooxyacetic acid hemihydrochloride (AOA) (C13408), were obtained from Sigma. U0126 (9903) was obtained from Cell Signaling, Torin1 (4247) was obtained from Tocris. Paclitaxel (P-9600) and Capecitabine (C-2799) were obtained from LC Laboratories. Antibodies against P-AMPK (Thr172), AMPK, HA-Tag, ACC, phospho-ACC (Ser79), LKB1, Rheb, TSC2, phospho-TSC2 (Ser1387), mTOR, Raptor, ULK1, phosphor-ULK1
(Ser555/Ser757), mTOR, Raptor, P-Rb (Ser780), Rb, Cyclin D1, Cyclin A2, Rictor, actin were obtained from Cell Signaling. Glucose-free DMEM was from Invitrogen (11966-025). Dialyzed fetal bovine serum (F0392) was obtained from Sigma-Aldrich. siRNAs targeting LKB1 (sc-35816), AMPK (sc-45312), Raptor (sc-44069), mTOR (sc-35409) were obtained from Santa Cruz Biotechnology; and siRNAs targeting Rheb (M-009692-02-0005), PLD1 (M-009413-00-0010), PLD2 (M-005064-01-0010) were obtained from Dharmacon. AICAR and compound C were obtained from Tocris Bioscience. PLD inhibitors for PLD1 (VU0379595) and PLD2 (VU0285655-1), and PA (1-palmitoyl 2-oleoyl), PS (1-palmitoyl-2oleoyl-sn-Glycero-3-Phospho-L-Serine) in chloroform were purchased from Avanti-Polaris Lipids. Antibody against PCNA and siRNA targeting Rictor (sc-61478), control siRNA were obtained from Santa Cruz Biotechnology. AICAR, FK506 was obtained from Tocris Bioscience.

2.3 Plasmids

Plasmids for transient transfections were obtained from the following sources: pcDNA3.1 control plasmid was obtained from Invitrogen. The plasmid expression vectors for HA-tagged catalytically inactive PLD1 and PLD2 (pCGN-PLD1-K898R) and pCGN-PLD2-K758R) were generous gifts from Dr. Michael Frohman (SUNY-Stony Brook, NY). Vector expressing mutant K-Ras (G12V) was obtained from Missouri S&T cDNA Resource Center, Rolla, MO, USA.

2.4 siRNA

Cells were plated on 6-well plates at 30% confluence in medium containing 10% serum. After 24 h, cells were transfected with siRNA at 100nM concentration using Lipofectamine.
RNAiMAX (Invitrogen) according to manufacturer’s instructions. After 6 h, the medium was changed to fresh medium containing 10% serum and 48 h later, cells were lysed and analyzed by Western blot.

2.5 Western Blot Analysis

Proteins were extracted from cultured cells in M-PER (Thermo Scientific, 78501). Equal amounts of proteins were subjected to SDS-PAGE on poly-acrylamide separating gels. Electrophoresed proteins were then transferred to nitrocellulose membrane. After transfer, membranes were blocked in an isotonic solution containing 5% non-fat dry milk in PBS. Membranes were then incubated with primary antibodies as described in the text. Depending on the origin of the primary antibody, either anti-mouse or anti-rabbit HRP conjugated IgG was used for detection using ECL system (Pierce). Relative protein level of AMPK phosphorylation were normalized to AMPK and quantified using LI-COR image studio software.

2.6 Flow cytometric analysis

Cultured cells were washed and trypsinized. Cell suspensions were recovered and resuspended in the following fixing solution: 7ml 1X phosphate buffered saline, 2% bovine serum albumin, 5mM EDTA, 0.1% NaN₃. 3ml of 100% ethanol was added drop wise. Fixed cells were centrifuged, washed, and then resuspended in 500μl sorting buffer: 1X phosphate buffered saline, 0.1% Triton-X 100, 2% bovine serum albumin, 5mM EDTA, 40μg/ml propidium iodide, 100μg/ml RNAse A, and incubated at 37C for 30 min. The cells were filtered through 70-μm mesh to remove cell aggregates. The DNA content was analyzed by flow cytometry (FACSCalibur; Becton Dickinson), and percentages of cells
within each phase of the cell cycle were determined using WinCycle software (Phoenix Flow Systems).

2.7 Cell proliferation assay

At indicated times, cells in six-well plates were washed once with PBS, trypsinized with 500 μl trypsin, resuspended in 500 μl complete medium. The cells were stained using 0.1% crystal violet solution (Sigma), and then counted twice using hemocytometer.

2.8 Trypan blue exclusion cell viability assay

At indicated time points post-treatment, floating and adherent cells were collected and pelleted by centrifugation. The cell pellet was resuspended in 500 μl PBS, stained using 500 μl 0.4% trypan blue dye (Sigma), and incubated at room temperature for 10 min. Both dead (stained) and live (unstained) cells were counted twice using hemocytometer, and percent non-viable cells was determined from dividing dead cells by total number of cells.

2.9 Preparation of PA

Immediately before use, the appropriate amount of PA was dried under nitrogen and resuspended by vortexing briefly in DPBS (SAFC Biosciences, 59321C). The lipid suspension was then sonicated in a water bath sonicator for 5 min. The resulting PA suspension was immediately added to cell culture to a final concentration of 300μM. Cells were lysed after 45 minutes of PA stimulation. PS preparation was also done by following same protocol mentioned above.
2.10 PLD Activity

PLD activity was determined by accumulation of the transphosphatidylation product $[^3\text{H}]$-phosphatidylbutanol as described previously (85). Lipid membranes were labeled with $[^3\text{H}]$-myristic acid (60 Ci/mmole; 1.5 μCi/ml; Perkin-Elmer) for 4 hours. 1-BtOH was added for 20 min before lipids where collected. Lipids were extracted and separated by thin layer chromatography along with phosphatidylbutanol standard (Enzo Life Sciences, BML-ST401-0050). The phosphatidylbutanol fraction was identified through co-migration with standards and the levels of the PLD product $[^3\text{H}]$-phosphatidylbutanol was determined by scintillation counting after scraping the phosphatidylbutanol band from thin layer chromatography plates. Error bars for PLD assays represent S.D. values for at least two independent experiments. $P$ values were calculated using a paired student’s $t$-test, two tailed.

2.11 Immunoprecipitation

Cells were grown in 10-cm-diameter plates. Immediately before lysing, culture plates were rinsed once with cold PBS and lysed on ice for 30 min in 500μl of ice-cold 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) immunoprecipitation buffer (40 mM HEPES [pH 7.5], 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 0.5 mM orthovanadate, protease inhibitors [Millipore]) containing 0.3% CHAPS. A 500μg sample of protein was then incubated with appropriate antibodies, and the immunoprecipitates were recovered 16 h later with protein G-Sepharose. The immunoprecipitates were then subjected to Western blot analysis along with 40μg of total cell lysate.
CHAPTER III

Blocking anaplerotic entry of glutamine to TCA cycle sensitizes K-Ras mutant cancer cells to cytotoxic drugs
3.1. Introduction

Metabolic dysregulation is an emerging hallmark in cancer.(1) Coupling oncogenesis with the needs of proliferative metabolism, several oncogenes that cause cellular transformation also upregulate glycolytic enzymes and promote metabolic reprogramming.(4,86) In order to meet increased anabolic demand, cancer cells display elevated levels of glucose uptake. However, instead of complete oxidation of glucose through the tricarboxylic acid (TCA) cycle, most cancer cells convert glucose to lactate through a process known as aerobic glycolysis.(87) This metabolic transformation was first described by Otto Warburg in the early 1920s and named Warburg effect.(88) It has been proposed that less efficient utilization of glucose for ATP generation is overcome by a marked increase in glucose uptake.(2) Another metabolic shift is the utilization of the TCA cycle intermediate citrate for cytosolic generation of acetyl-CoA. After conversion of the glycolytic product pyruvate to acetyl-CoA in the mitochondria, there is a condensation reaction with oxaloacetate to generate citrate, which exits the mitochondria where it is converted back to oxaloacetate and acetyl-CoA, which can then be used for fatty acid synthesis. This creates a need for anaplerotic replenishment of TCA cycle intermediate that can regenerate oxaloacetate. The most common source for anaplerosis is glutamine (Q), which can be successively deaminated in two steps to produce α-ketoglutarate – allowing for the maintenance of TCA cycle function.(4) The Myc oncogene has been shown to upregulate glutaminolysis leading to Q addiction in cancer cells.(89,90) While Q has been reported to play pleiotropic roles in tumor proliferation, the impact of Q deprivation on cancer cell cycle progression is less well characterized.(91,92) This is further complicated by the differential response of cancer cells to Q deprivation, which
likely depends on the mutations they harbor. For instance, cancer cells with Myc overexpression undergo apoptotic cell death in response to Q depletion. (93) On the other hand, in K-Ras overexpressing NIH 3T3 mouse fibroblasts, Q deprivation was shown to cause abortive S-phase. (9) Additionally, we recently reported that some cancer cell lines bypass a Q-dependent G1 cell cycle checkpoint and arrest in S- and G2/M-phase of the cell cycle upon Q deprivation. (12)

3.1Purpose of the study in this chapter

In this report, we demonstrate that cancer cells harboring K-Ras mutations arrest in S- and G2/M-phase of cell cycle rather than G1. Significantly we also show that this differential sensitivity to Q in K-Ras mutant cancer cells can be exploited using cell cycle phase specific cytotoxic drugs. Our study provides proof-of-principle that cancers with specific genetic defects and dysregulated metabolic cell cycle checkpoints can create a synthetic lethality to chemotherapeutic drugs and offer novel therapeutic options.

3.2Results

3.2.1 Glutamine deprivation causes S- and G2/M-phase arrest

K-Ras mutant cancer cells

Glutamine deprivation causes G1 cell cycle arrest in non-transformed primary cells (12). We previously reported that MDA-MB-231 breast and Panc-1 pancreatic cancer cell lines fail to arrest in late-G1 upon Q or essential amino acid deprivation. (12) We therefore screened several cancer cell lines to identify underlying genetic mutations that override the Q-mediated G1 checkpoint. As seen in Figure 3.1A, Q deprivation for 48 hr caused
significant accumulation of cells in G1 phase at the expense of S- and G2/M-phase cells in MCF7 breast, and DU-145 and LNCaP prostate cancer cell lines. On the other hand, Q deprivation led to an increase primarily in S-phase cells and a reduction in G1-phase cells in MDA-MB-231 breast, PANC-1 pancreatic, and Calu-1 lung cancer cells. The cell lines that failed to arrest in G1 upon Q deprivation all harbor oncogenic K-Ras mutations (Figure 3.1A). The failure to arrest in G1 upon Q deprivation in these K-Ras mutant cancer cell lines was neither tissue specific nor K-Ras mutation site specific (Figure 3.1A).

Since amino acid deprivation has been implicated in mTOR signaling, we also evaluated the impact of Q deprivation on mTOR substrate phosphorylation. While Q deprivation caused a modest reduction in S6 kinase phosphorylation, there were no significant differences between the cells that arrested in G1 and those that arrested in S- and G2/M-phase (Figure 3.2). However, there were elevated levels of Akt phosphorylation at Ser473 and Thr 308 observed with Q deprivation in the mutant K-Ras-driven cancer cells that is concomitant with non-G1 arrest. This observation is consistent with a recent report that phosphorylation of Akt occurs predominantly as cells progress into S-phase.(94) We also looked at the impact of Q deprivation on the cell cycle progression markers cyclins D, E, A, and B, phospho-Rb, and p27 protein levels. As shown in Figure 3.2, Q deprivation in the cancer cells with wild type K-Ras had very little impact on anything other than cyclin B, which was lower in the Q-deprived cells. In the cells with mutant Ras, there were subtle reductions in cyclins D and E, phospho-Rb, and p27 levels, with a concomitant increase in S-phase marker cyclin A. The reduction in G1 cell cycle markers in K-Ras mutant cancer cells is consistent with a non-G1 arrest observed by FACS analysis (Figure 3.1A); however, they do not provide insight into how K-Ras-driven cancer
cells avoid G1 arrest. To establish that Q deprivation was not merely prolonging S-phase and thereby causing an increase in the percentage of cells in S-phase, we performed cell proliferation assay. In all the cell lines tested, there was a significant loss of cell proliferation upon Q deprivation (Figure 3.1B). The data in Figure 3.1 reveal a correlation between dysregulated Q-mediated G1 cell cycle arrest and K-Ras mutation.

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Figure 3.1. Glutamine deprivation causes S- and G2/M-phase arrest in K-Ras mutant cancer cells. A, MCF7 breast, DU-145 prostate, LNCaP prostate, MDA-MB-231 breast Panc-1 pancreatic, and Calu-1 lung cancer cells (obtained from the American Type Culture Collection, Manassas, VA, USA) were plated at 30% confluence in 10-cm plates in complete media (CM) containing 10% serum. After 24 hr, the cells were shifted to CM or medium lacking Q for 48 hr. Both CM and –Q medium contained 10% dialyzed FBS (F0392, Sigma-Aldrich, St. Louis, MO, USA). After 48 hr, the cells were harvested and analyzed for cell cycle distribution by measuring DNA content/cell as described previously.(12) The error bars represent standard error of mean for experiments repeated four times. The mutations present in the cancer cell lines were obtained from the Sanger Institute COSMIC database (95) and the Cancer Cell Line Encyclopedia.(96) B, Cells were plated at 20% confluence in six-well plates in CM containing 10% serum. After 24 hr (day 1), cells were shifted to CM or medium lacking Q. Cells were harvested at indicated time points, stained using crystal violet, and quantified by light microscopy. Error bars represent the standard error of the mean for experiments repeated three times.
Fig 3.2: Effect of glutamine deprivation on PI3K/mTOR pathway and cell cycle regulatory proteins in K-Ras WT and mutant cancer cell lines. A, Cells were plated at 30% confluence in 10-cm plates in complete media containing 10% serum for 24 hr, at which time they were shifted to CM or medium lacking Q for 4 hr. The cells were subsequently harvested and cell lysates were collected. The indicated protein or phosphoprotein levels were determined by Western blot analysis. B, The cells were plated and treated as in A for 48 hr, at which time cells were harvested, lysed, and analyzed for protein levels using Western blot. The data shown are representative of experiments repeated two times.

3.2.2 Override of Q-dependent G1 checkpoint requires activation of multiple signaling pathways

Based on elegant studies by Weinberg and colleagues on the minimal genetic requirements for the transformation of human cells, (97,98) we have proposed that
cooperating genetic mutations in human cancer cells impact on signaling pathways that lead to passage through two major G1 cell cycle checkpoints(99) that have both been referred to as the restriction point.(100) The first checkpoint is in mid-G1 and is dependent on growth factors and facilitates passage through a checkpoint regulated by cyclin D and ERK; the second checkpoint is dependent on nutrients (including Q) and is regulated by cyclin E and mTOR.(12) Interestingly, both of these pathways can be activated by mutant K-Ras (Figure 3.3A).(101,102) To investigate if either or both of these key regulatory pathways are critical for overriding the Q-dependent G1 checkpoint, we investigated whether we could restore G1 arrest in response to Q deprivation by pharmacological suppression of the Raf/Mek/ERK and the phosphatidylinositol-3-kinase (PI3K)/Akt/mTOR (mammalian target of rapamycin) pathways (Figure 3.3A). We evaluated the effect of U0126 (Mek inhibitor) and Torin1 (mTOR inhibitor) on Q-induced cell cycle arrest in MDA-MB-231 and Panc1 cells. As shown in Figure 3.3B, U0126 and Torin1 suppressed the phosphorylation of the Mek and mTOR substrates ERK and S6 kinase respectively in both the MDA-MB-231 and Panc1 cells. As shown in Figure 3.3C, in the absence of Q, S-phase arrest was observed as in Figure 3.1 in the MDA-MB-231 cells. Neither U0126 nor Torin1, by itself, reverted the cells to G1 arrest upon Q deprivation. However, treatment with U0126 and Torin1 together did revert the MDA-MB-231 cells to G1 arrest in the absence of Q (Figure 3.3C). Similar results were obtained when we used the PI3K inhibitor wortmannin instead of Torin1 (Figure 3.3G). We also evaluated the impact of U0126 and Torin1 on Q-induced cell cycle arrest in the Panc-1 cells, which arrested in both S- and G2/M phase. Unlike the MDA-MB-231 cells, the Panc-1 cells were largely reverted to G1 arrest with only Torin1. U0126 did not do much
by itself and marginally improved G1 arrest when combined with Torin1 (Figure 3.3D). The Panc1 cells interestingly still remained arrested in G2/M when treated with Torin1 – indicating a differential mechanism for G2/M arrest for the MDA-MB-231 and Panc1 cells.

We next examined the MCF7 cells, which do not have a K-Ras mutation and arrest in G1 in response to Q deprivation. These cells were treated with the tumor-promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), which has been shown to stimulate the Raf/Mek/ERK pathway,(103) which is commonly activated by mutant K-Ras. As shown in Figure 3.3E, TPA treatment caused a shift from G1 to S-phase arrest in response to Q deprivation. This change in arrest pattern could be reverted to G1 arrest with U0126, but not with Torin1 – indicating that it was activation of the ERK pathway that was responsible.

The data in Figures 3.1 and 3.3E reveal a correlation between K-Ras mutation or activation of the Raf/Mek/ERK pathway and dysregulated G1 cell cycle progression through a Q-dependent checkpoint. We therefore investigated whether mutant K-Ras alone is sufficient to override the Q-dependent G1 checkpoint. We introduced a vector that expresses mutant K-Ras (G12V) into the immortalized human diploid fibroblast cell line BJ-hTERT(104) and evaluated the impact of Q deprivation on cell cycle progression. As shown in Figure 3.3F, mutant K-Ras, by itself, failed to override G1 cell cycle arrest – indicating that additional genetic alterations in the cancer cells are required for dysregulating the Q-dependent G1 checkpoint. However, mutant Ras has also been reported to induce senescence in primary cells.(105) Weinberg and colleagues reported previously that mutant Ras in combination with TPA induces a transformed phenotype in primary cells.(106) We therefore investigated the effect of mutant K-Ras in combination
with TPA on cell cycle progression when cells were depleted of Q. As shown in Figure 3.3F, the combination of mutant K-Ras and TPA resulted in S- and G2/M-phase arrest upon Q deprivation rather than G1. Collectively, the data in Figure 3.3 reveal that while mutant K-Ras, by itself, is not sufficient to cause override of the Q-dependent G1 cell cycle checkpoint, override can be achieved by cooperating genetic alterations or by activation of a cooperating signaling pathway.
Figure 3.3. Override of Q-dependent G1 checkpoint requires activation of multiple signaling pathways. A, Schematic diagram of the Raf /Mek /ERK and PI3K/Akt/mTOR signaling pathways that regulate distinct G1 cell cycle checkpoints.(12) B, MDA-MB-231 and Panc1 cells were plated at 30% confluence in 10-cm plates in CM containing 10% serum. After 24 hr, the cells were treated with 10 µM U0126 (Cell Signaling Technology, Danvers, MA, USA) and/or 250 nM Torin1 (Tocris Bioscience, Bristol, UK) as indicated for 4 hr at which time cell lysates were prepared and used for Western blot analysis of the levels of phospho-Akt (S473), phospho-S6 kinase (S6K) (T389), phospho-ERK1/2 (T202/Y204), Akt, S6K, ERK1/2, and actin (antibodies all from Cell Signaling Technology). The data shown is representative of experiments repeated at least two times. C-D, MDA-MB-
231 and Panc1 cells were plated as in B. After 24 hr, the cells were shifted to CM or medium lacking Q and treated with 10 µM U0126 and/or 250 nM Torin1 for 48 hr. The cells were then analyzed by flow cytometry as in Figure 1. Error bars represent standard error of mean for the experiment repeated four times. E, MCF7 cells were prepared as in C-D, and evaluated similarly as for the MDA-MB-231 and Panc1 cells except that cells were also treated with 100 nM TPA (Cell Signaling Technology) as indicated concomitantly with U0126 or Torin1. F, BJ-hTERT human diploid fibroblasts (obtained from ATCC) were transfected with either mock control or a vector expressing mutant K-Ras (G12V; obtained from Missouri S&T cDNA Resource Center, Rolla, MO, USA) using PolyFect transfection reagent (Qiagen, Germantown, MD, USA) per manufacturer’s recommendations. K-Ras G12V overexpression and activation of downstream phospho-ERK was confirmed by Western blot analysis. The cells were then put in either complete medium (CM) or in medium lacking Q for 48 hr, at which time cell cycle status was determined as in Figure 3.1A. TPA (100 nM) was added where indicated at the time of Q withdrawal. Error bars represent standard error of mean for experiments repeated three times. G, MDA-MB-231 cells were plated at 30% confluence in 10 cm plates in CM containing 10% serum. After 24 hr., the cells were shifted to CM or media lacking Q and treated with 10uM U0126 (Cell Signaling Technology, Danvers, MA, USA) and/or 1uM Wortmannin (Sigma-Aldrich) for 48 hr. The cells were analyzed by flow cytometry as in Fig 3.1A. Error bars represent standard error of mean for the experiment repeated two times.

3.2.3 Glutamine deprivation sensitizes K-Ras mutant cancer cells to phase-specific cytotoxic drugs

We next examined whether the differential cell cycle arrest observed with Q deprivation could create synthetic lethality to phase specific cytotoxic drugs in K-Ras mutant cancer cells. To specifically target the S-phase arrested cells, we used
capecitabine, a pro-drug that is converted enzymatically to 5-fluorouracil and elicits a DNA damage response leading to apoptosis.\((107)\) To target G2/M-phase arrested cells, we used paclitaxel, which stabilizes microtubules and induces apoptotic cell death in the mitotic phase.\((108)\) As seen in Figures 3.4A-D, Q deprivation alone led to a modest increase in nonviable cells in both K-Ras wild type and K-Ras mutant cancer cell lines. In the K-Ras wild type cell lines, addition of capecitabine or paclitaxel to Q-deprived cells did not cause significant increases in non-viable cells compared to controls (Figures 3.4A and 3.4B). However, in the K-Ras mutant MDA-MB-231 and PANC-1 cells, Q deprivation followed by treatment with either capecitabine or paclitaxel caused a significant increase in the nonviable cells (Figures 3.4C and 3.4D). Thus, in K-Ras mutant cancer cell lines, Q deprivation creates a synthetic lethality for the cell cycle phase specific cytotoxic drugs. There was a somewhat elevated background of cell death with Q deprivation in the MDA-MB-231 cells, but there are very stark differences between the sensitivity of the cells harboring K-Ras mutations with those that do not.

As shown in Figure 3.3, we were able to get MCF7 cells and BJ cells expressing mutant K-Ras to bypass the G1 Q-dependent cell cycle checkpoint and arrest in S- and/or G2/M phases when treated with TPA. We therefore examined whether these cells became sensitive to capecitabine and paclitaxel. As shown in Figure 3.4E and 3.4F, both MCF7 and BJ-K-Ras cells, upon Q depletion, became sensitive to capecitabine and paclitaxel when treated with TPA. Thus, it is the arrest in S and G2/M that sensitizes cells to capecitabine and paclitaxel.
Figure 3.4 Glutamine deprivation sensitizes K-Ras mutated cancer cells to phase-specific cytotoxic drugs. MCF7 A, DU-145 B, MDA-MB-231 C, and Panc1 D, cells were plated at 20% confluence in six-well plates in complete media (CM) containing 10% serum for 24 hr, after which the cells were shifted to CM or medium lacking Q.
for 48 hr. After 48 hr, the cells were additionally treated with 50 nM paclitaxel (Pac) or 1 µg/ml capecitabine (Cap) (both from Sigma-Aldrich) for 24 hr. Percent cell viability was determined using trypan blue dye exclusion assay as described previously.(109) MCF7 E and BJ-K-Ras F, cells were placed in CM or medium lacking Q for 48 hr in the presence and absence of 100 nM TPA as indicated. After 48 hr, the cells were additionally treated with paclitaxel and capecitabine for 24 hr and cell viability was determined as in (A-D). Error bars represent the standard error of the mean for experiments repeated three times.

3.2.4 Pharmacological inhibition of Q anaplerosis sensitizes K-Ras mutant cancer cells to cytotoxic drugs

Glutamine, via anaplerotic entry to the TCA cycle, replenishes the intermediates lost by the exit of citrate from the mitochondria for fatty acid and cholesterol biosynthesis.(5) Glutaminase catalyzes the deamination of Q to generate glutamate. Glutamate can then be converted to α-ketoglutarate by either glutamate dehydrogenase or transaminase. Kimmelman and colleagues recently reported that K-Ras-driven pancreatic cancer cells preferentially utilize the transaminase pathway for anaplerotic glutamine utilization.(11) In the transaminase pathway, glutamate acts as an amino donor to oxaloacetate – a reaction catalyzed by glutamate oxaloacetate transaminase (GOT), which generates aspartate and α-ketoglutarate (schematic shown in Figure 3.5A). Anaplerotic entry of Q into the TCA cycle can be inhibited by aminoxyacetate (AOA) – a pan-transaminase inhibitor, which inhibits GOT and consequently the entry of glutamine into the TCA cycle.(110,111) Treatment of both MCF-7 and MDA-MB-231 cells with AOA for 48 hr led to morphological changes similar to that observed with Q deprivation (Figure
3.5B). As seen in Figure 3.5C, AOA treatment caused G1 arrest in the MCF-7 cells and S- and G2/M-phase arrest in the MDA-MB-231 cells and also blocked proliferation (Figure 3.5D) as was observed with Q deprivation in Figure 3.5A and B – indicating that AOA mimics Q deprivation in both cell types. We also investigated whether the effect of AOA on cell cycle progression could be reversed by providing cell permeable analogues of α-ketoglutarate and aspartate – the products of the transamination reaction between glutamate and oxaloacetate. Dimethyl-α-ketoglutarate (DMKG) and β-methyl-aspartate (β-MD) were included along with AOA for the MDA-MB-231 cells. As shown in Figure 3.5E, neither compound by itself was able to completely reverse S- and G2/M arrest seen in the MDA-MB-231 cells, however the combination of both DMKG and β-MD did reverse the S- and G2/M arrest in these cells – indicating that generating both α-ketoglutarate and aspartate in the transaminase reaction is critical for passing through S- and G2/M phases. This finding is similar to that observed by Kimmelman and colleagues who showed a requirement for both α-ketoglutarate and non-essential amino acids (which included aspartate) for colony formation by pancreatic cancer cells. The need for aspartate as well as α-ketoglutarate indicates that the aspartate generated by the transaminase reaction is important. Kimmelman and colleagues (11) demonstrated a critical need for conversion of aspartate → oxaloacetate, followed by conversion of oxaloacetate → malate, and then oxidative decarboxylation to pyruvate by malic enzyme in order to generate NADPH and maintain redox balance. However, a substantial amount of anaplerotic Q is converted to fatty acids. (87) Thus, the aspartate generated by the transaminase reaction between glutamate and oxaloacetate is likely destined to re-enter
the TCA cycle via conversion to oxaloacetate followed by condensation with acetyl-CoA to form citrate (see Figure 3.5A).

We further investigated whether AOA treatment would mimic Q deprivation to create the synthetic lethality observed with cell cycle phase-specific cytotoxic drugs in K-Ras mutant cancer cells. AOA treatment by itself led to minimal increase in nonviable cells in both MCF-7 and MDA-MB-231 cells (Figure 3.5F). AOA treatment alone also did not induce significant increases in cleaved PARP (Figure 3.5F), an indicator of apoptosis – suggesting that AOA has low cytotoxicity. This is important in that AOA also inhibits alanine transaminase as well.(11) Similar to what was observed with Q deprivation, the combination of AOA and the cytotoxic drugs did not increase the percentage of nonviable cells and cleaved PARP levels in MCF-7 cells (Figure 3.5D). However, the combination of AOA and the cytotoxic drugs caused a significant increase in nonviable cells and cleaved PARP levels in MDA-MB-231 cells (Figure 3.5D). These data indicate that in K-Ras mutant cancer cells, pharmacological inhibition of anaplerotic entry of Q into the TCA cycle mimics Q deprivation to produce aberrant cell cycle arrest creating synthetic lethality to cell cycle phase specific cytotoxic drugs.

Metabolic transformation is an emerging paradigm in cancer biology(1). Glutamine has been suggested to play pleiotropic roles in tumor proliferation, and several oncogenes have been shown to promote aerobic glycolysis and Q addiction in cancer cells.(7,92) We previously reported that while most cells arrest in late G1 in response to Q deprivation, some cancer cell lines arrested in S and G2/M.(12) An extension of this study revealed a correlation between G1 Q-checkpoint override in human cancer cell lines with K-Ras mutations. Upon Q deprivation, cancer cell lines harboring K-Ras mutations arrested in
S and G2/M rather than G1. Whether there are actual Q-sensitive checkpoints in S- and G2/M phases is not clear from data presented here, however it is clear from Figure 3.1B that cell number is not increasing and therefore cells are stopping progression in S and G2/M phases and it is here that they are sensitive to cytotoxic drugs. Of significance, Q deprivation created a synthetic lethality for compounds that selectively target cells in S and G2/M phases of the cell cycle. Importantly, Q deprivation could be mimicked with AOA – a compound that interferes with the transaminase pathway for anaplerotic conversion of Q into the TCA cycle intermediate α-ketoglutarate. The ability of AOA to mimic Q deprivation is consistent with the recent report that K-Ras-driven pancreatic cells preferentially use the transaminase over the glutamate dehydrogenase pathway for generating α-ketoglutarate. Thus, it is possible that a combination of drugs that block the generation of α-ketoglutarate via transamination and kill cells in S- and/or G2/M phase could be used to target K-Ras driven cancers (Figure 3.5G).
Figure 3.5 Pharmacological inhibition of Q anaplerosis sensitizes K-Ras mutant cancer cells to cytotoxic drugs. A, Schematic overview of anaplerotic Q utilization. Q is deaminated to glutamate by glutaminase (GLS). Glutamate is then converted...
to α-ketoglutarate via transamination catalyzed by GOT, which uses oxaloacetate as the amino group acceptor to generate aspartate. AOA inhibits GOT and therefore suppresses generation of α-ketoglutarate from Q-derived glutamate. Aspartate is critical for redox balance and NAPDH production and the generation of citrate for fatty acid synthesis. B, Cells were plated at 20% confluence in 10-cm plates in complete medium (CM). After 24 hr, cells were shifted to CM, or medium lacking Q, or CM containing 0.5 mM AOA (C13408, Sigma-Aldrich) for 48 hr – at which time the cells were observed using phase-contrast microscopy. C, MCF7 and MDA-MB-231 cells were plated and treated as in (b) for 48 hr, at which time cells were analyzed for cell cycle distribution as in Figure 1. In addition to AOA, the MDA-MB-231 cells were treated with cell permeable analogues of α-ketoglutarate (DMKG; 4 mM) (349631, Sigma-Aldrich) and aspartate (β-MD; 4 mM) (A8921, Sigma-Aldrich). Error bars represent standard error of mean for experiments repeated three times. D, Cells were plated and treated as in B, harvested at indicated time points, and scored after staining with crystal violet using light microscopy. Error bars represent the standard error of the mean for experiments repeated three times. E, MCF7 and MDA-MB-231 cells were plated as in B, and shifted to CM or treated with 0.5 mM AOA for 48 hr. The cells were additionally treated with 50 nM Pac or 1 µg/ml Cap for 24 hr, at which time the percentage non-viable cells were determined using trypan blue exclusion assay. Error bars represent the standard error of mean for experiments repeated three times. Cell lysates were also collected, and the levels of cleaved PARP (antibody from Cell Signaling) were determined by Western blot analysis. Data shown are representative of experiments repeated two times. F, Model depicting that AOA treatment mimics Q deprivation causing G1 cell cycle arrest in K-Ras wild type cells and S- and G2/M-phase arrest in K-Ras mutant human cancer cell lines, which creates synthetic lethality to cell cycle phase-specific cytotoxic drugs causing apoptotic cell death.
3.3 Discussion

While there was a correlation between cancer cells harboring K-Ras mutations and override of the Q-dependent G1 cell cycle checkpoint, K-Ras, by itself, was not sufficient to induce override of the Q-dependent G1 checkpoint in immortalized BJ cells. However, the inability to observe bypass of the Q-dependent G1 checkpoint was likely due to the induction of senescence by mutant K-Ras.(105) However, if TPA, which cooperates with mutant Ras to transform primary cells(106) was provided, the cells bypassed the G1 checkpoint and arrested in S- and G2/M phases and were now sensitive to capecitabine and paclitaxel. In addition, we observe override of the Q-dependent G1 cell cycle checkpoint by activating the ERK pathway in MCF-7 cells – indicating that bypass could be achieved by activating this K-Ras effector pathway. Panc-1 pancreatic cancer cells that harbor a K-Ras mutation arrested in S- and G2/M phase with Q deprivation, but could largely be reverted to G1 arrest with only mTOR suppression. Thus, it is clear that override of the Q-mediated G1 checkpoint is complex involving more than one signaling pathway. Combined inhibition of two key regulators of G1 cell cycle progression – ERK and mTOR – reverted override of the Q-mediated G1 checkpoint, indicating that override is dependent on two key signaling nodes implicated at two distinct regulatory G1 checkpoints that have both been referred to as the restriction point.(12) Whether this phenomena is absolutely dependent on the K-Ras or K-Ras effector signals is not clear – nor is it clear that all cancer cells with K-Ras mutations will override the Q-dependent checkpoint. In fact the DU-145 prostate cancer cells used in this study harbors an unusual UBE2L3-KRas fusion protein, knockdown of which was shown to attenuate cell invasion and xenograft growth(112), but still arrested in G1 in response to Q deprivation as seen
in Figure 3.1a. Thus, while the connection between activated K-Ras and override of the Q-dependent G1 checkpoint is not likely absolute, the correlation observed here with human cancer cell lines harboring K-Ras mutations and a synthetic lethal sensitivity to paclitaxel and capecitabine suggests novel strategies for therapeutic intervention in a class of cancers considered undruggable.

To test the hypothesis that Gln deprivation in K-Ras-driven cancer cells could create sensitivity to cytotoxic compounds, we deprived K-Ras-driven cancer cells of Gln and examined sensitivity to capecitabine, which interferes with DNA synthesis; and paclitaxel, which interferes with microtubule breakdown during mitosis. Both capecitabine and paclitaxel induced apoptosis in K-Ras-driven, but not in cancer cells lacking K-Ras mutation that arrested in G1 upon Gln depletion. Clearly, Gln deprivation is not a viable therapeutic option; however, interfering with anaplerotic utilization of Gln is possible.(113) Kimmelman and colleagues recently reported that Gln is utilized in K-Ras-driven pancreatic cancer cells via a transamination reaction whereby glutamate is deaminated to α-ketoglutarate with concomitant generation of aspartate from oxaloacetate (11). Thus, in K-Ras-driven cancer cells, the transaminase pathway is apparently preferred over the glutamate hydrogenase pathway, which is used when glucose levels are low.(113) Consistent with findings reported by the Kimmelman group, we found that the transaminase inhibitor aminooxyacetate (AOA) mimicked Gln depletion and created sensitivity to capecitabine and paclitaxel in K-Ras-driven cancer cells. Thus, a combination of transaminase inhibitors along with capecitabine and paclitaxel could be a viable strategy for treating K-Ras-driven cancers that exploits the ability of K-Ras to stimulate override of the Gln-dependent G1 checkpoint. This approach represents a
“synthetic lethal” situation(114) whereby activating K-Ras mutations in combination with suppressed Gln utilization sensitizes cells to the cytotoxic effects of cell cycle phase-specific effects of capecitabine and paclitaxel.

Thus, after receiving growth factor signals indicating that it is appropriate to divide, there are perhaps several late G\textsubscript{1} metabolic checkpoints that monitor whether there are sufficient nutrients available for the cell to double its mass and divide.(115) This is shown schematically in Figure 3.6. Importantly, cancer cells harboring K-Ras mutations did not arrest in G\textsubscript{1} upon Gln deprivation. Instead, K-Ras-driven cancer cells progress into S- and G\textsubscript{2}/M-phase where they arrested.(116) Thus, mutant K-Ras confers the ability to override the Gln-dependent late G\textsubscript{1} checkpoint allowing progression from G\textsubscript{1} into S-phase in the absence of Gln. Suppression of the K-Ras downstream effectors mTOR and Erk restored G1 arrest in response to Gln deprivation in K-Ras-driven cancer cells – indicating that override of the G1 Gln checkpoint was mediated by activation of mTOR and Erk. Cells that have committed to divide and progress into S and G\textsubscript{2}/M are, in general, more vulnerable to apoptotic insult. Thus, K-Ras-driven cancer cells, which override a Gln-dependent G\textsubscript{1} cell cycle checkpoint and arrest in S and G\textsubscript{2}/M, could be sensitive to therapeutic strategies that deprive cells of Gln and target cells in S and G\textsubscript{2}/M.
Figure 3.6 Schematic overview of late G1 metabolic cell cycle checkpoints Adopted from (10): The relative positions of the growth factor-dependent Restriction Point and late G1 metabolic checkpoints mediated by essential amino acids, Gln and mTOR are depicted.

Oncogenic K-Ras mutations are common in many different cancer types and contribute to as many as 30% of all human cancers – including 90% of pancreatic cancers (117) - which have a poor prognosis with mortality usually within 6 months of detection and a five-year survivability of less than 5% (118). K-Ras mutants have enhanced binding to GTP and are considered undruggable – due in part to the picomolar range for GTP binding (119). Thus, targeting K-Ras-driven cancers has been problematic – especially for pancreatic cancer, which is likely the deadliest of human cancers. The observation reported here – that inhibition of anaplerotic utilization of Q in K-Ras-driven human cancer cell lines leads to cell cycle arrest in S- and G2/M rather than G1 – suggests a vulnerability for K-Ras-driven cancers that could be exploited therapeutically. We have demonstrated here an enhanced sensitivity to chemotherapeutic agents that
target cells in S- and G2/M – providing proof-of-principle for the predicted synthetic lethality created by interfering with Q utilization.

An emerging pattern for Ras-driven tumorigenesis involves changes in nutrient utilization as reported here for glutamine and previously by Kimmelman and colleagues.\(^\text{(11)}\) In addition, it was recently reported that Ras-driven cancer cells also have special needs for extracellular lipids \(^\text{(85,120)}\) and glutamine.\(^\text{(121)}\) Significantly, the need for exogenously supplied lipids in Ras-driven cancer cells creates a synthetic lethality for rapamycin.\(^\text{(85)}\) Thus, it is conceivable that the altered nutritional and metabolic needs by Ras-driven cancers may actually prove to be an Achilles’ heel for this deadly class of cancers.
CHAPTER IV

Reciprocal Regulation of AMP-activated Protein Kinase and Phospholipase D
4.1. Introduction

AMP-activated protein kinase (AMPK) is a critical signaling node that responds to cellular energy levels and alters cell metabolism accordingly (76,122). Altered metabolism has become a hallmark of cancer (1); and accordingly, AMPK is suppressed in many cancers (76). The best characterized mechanism for suppressing AMPK activity in human cancers is through mutations to the gene encoding liver kinase B1 (LKB1) (77). LKB1 had previously been reported to be a tumor suppressor associated with an inherited tumor susceptibility known as Peutz-Jeghers syndrome (123,124). The discovery of the connection between LKB1 and AMPK (82,84,125) clearly implicated altered metabolism in cancer. In response to elevated AMP/ATP ratios, AMPK binds AMP and then gets phosphorylated by LKB1. This leads to activated AMPK and the suppression of anabolic metabolism and a concomitant shift to catabolic events that generate ATP to restore energy homeostasis (76,126).

In addition to causing a shift from anabolic to catabolic metabolism, AMPK also stimulates a p53-dependent G1 cell cycle arrest (127,128). The LKB1/AMPK pathway suppresses mTOR (mammalian/mechanistic target of rapamycin) signaling (129,130). Suppression of mTOR also results in G1 cell cycle arrest (12). A key downstream target of AMPK is the tuberous sclerosis complex (TSC) consisting of TSC1 and TSC2. The TSC has a GTPase activating protein (GAP) activity towards the GTPase Ras homolog enriched in brain (Rheb) (46). AMPK phosphorylates TSC2 and activates its GAP activity resulting in suppressed Rheb due to the hydrolysis of bound GTP to GDP. Rheb is a key regulator of mTOR complex 1 (mTORC1) (131). Thus mechanistically, activating AMPK could cause cell cycle arrest by suppressing mTOR. Of interest is that while GTP-bound
Rheb is required for mTOR activation, Rheb binds mTOR in a GTP-independent manner (132). However, Rheb binds to, and activates, phospholipase D1 (PLD1) in a GTP-dependent manner (56). PLD generates the metabolite phosphatidic acid (PA), which is required for the stability and activity of the mTOR complexes mTORC1 and mTORC2 (54,58). PLD activity is required for the stimulation of mTOR by growth factors (72) and amino acids (55,57). Thus, a key role for Rheb could be the generation of PA needed for mTOR activity. PLD activity is commonly elevated in human cancer cells and, like mTOR, promotes cell cycle progression and survival (71,72).

4.1.1 Purpose of the study in this chapter

While the negative impact of AMPK on mTOR has been previously reported (129), much less is known about the impact of mTOR on AMPK signals. We report here that AMPK suppresses PLD activity in human cancer cells with elevated PLD activity and that PLD and PA suppress AMPK in an mTOR-dependent manner. This study reveals a means for cancer cells to suppress AMPK in order to promote cell proliferation.

4.2 Results

4.2.1 Activation of AMPK suppresses PLD activity

To investigate a connection between PLD and AMPK activities we examined the effect of glucose deprivation, which is known to elevate AMPK activity, on PLD activity. We used two K-Ras driven human cancer cell lines, MDA-MB-231 breast and Calu-1 lung, which we have reported previously to have elevated PLD activity (133,134). As expected, glucose deprivation resulted in elevated phosphorylation of AMPK at the LKB1 site at T172 in both the MDA-MB-231 and Calu-1 cells (Fig. 4.1A). As shown in Fig. 4.1A,
glucose deprivation also led to a suppression of the PLD activity in both MDA-MB-231 and Calu-1 cells. These data reveal a correlation between elevated AMPK activity and suppressed PLD activity.

AMPK can be activated by AICAR (5-aminoimidazole-4-carboxamide ribonucleoside) – a cell-permeable nucleoside that is metabolically converted to 5-aminoimidazole-4-carboxamide ribonucleoside monophosphate or ZMP by adenosine kinase. ZMP mimics the allosteric effects of AMP on the AMPK system (135). As shown in Fig. 4.1B, AICAR treatment activated AMPK as indicated by increased levels of phosphorylated AMPK at the LKB1 site. Also shown is the AICAR-induced increase in the level of phosphorylation of acetyl-CoA carboxylase (ACC) - a substrate of AMPK that regulates the synthesis of fatty acids. We next examined the effect of AICAR on PLD activity, and as shown in Fig. 4.1C, AICAR Suppressed the PLD activity in both MDA-MB-231 and Calu-1 cells. PLD catalyzes the hydrolysis of phosphatidylcholine to generate PA. We therefore examined the effect of AICAR on MDA-MB-231 and BJ-hTERT cells treated with PA. Of significance, the AICAR-induced increase in the phosphorylation of both AMPK and ACC was suppressed by PA treatment (Figs. 4.1D and 4.1F) whereas Phosphatidylserine, as a control lipid addition (Fig. 4.1E) can’t suppress the activation of AMPK upon AICAR treatment. These data further demonstrate that elevated AMPK activity suppresses PLD activity and intriguingly suggest that PLD generated PA prevents AMPK activation. We also investigated the regulation of AMPK while PLD activity is stimulated by phorbol esters, a known activator of PLD. TPA treatment along with AICAR diminished the activation of AMPK in MDA-MB-231 cells (Fig. 4.1G). This data further suggests that PLD generated PA prevents the AMPK activation.
Figure 4.1 Activation of AMPK suppresses PLD activity. A, MDA-MB-231 and Calu-1 cells were plated at 80% confluence in 60mm plates in DMEM containing 10% serum. 24 h later the cells were shifted to medium containing either complete DMEM or DMEM lacking glucose. Both complete DMEM and DMEM without glucose contained 10% dialyzed fetal bovine serum. After 4 hr, cells were harvested and lysates were prepared and used for Western blot analysis for the levels of phospho-
AMPK (P-AMPK), AMPK and actin. For PLD activity assay [³H]-myristic acid was added for 4 h in fresh medium to label lipids. 1-BtOH was added for 20 minutes, and the amount of the PLD-catalyzed transphosphatidylolation product, phosphatidyl-butanol, was determined as described under “Experimental Procedures”. Values were normalized to the levels of PLD activity in controls, which were given a value of 100%. B, MDA-MB-231 cells were plated as in A and 24 h later AICAR (2mM) was added for indicated times. Cells were harvested and the levels of P-AMPK, AMPK, phospho-ACC (P-ACC), ACC and actin were determined by Western blot analysis. C, MDA-MB-231 and Calu-1 cells were plated as in A and AICAR was added for 45 min. The cells were then harvested and the PLD activity was determined as in A. D, MDA-MB-231 cells were plated as in B. Where indicated, cells were treated with AICAR and/or (PA 300μM) for 45 min. The cells were then harvested and levels of P-AMPK, AMPK, P-ACC, ACC and actin were determined as in B. E, MDA-MB-231 cells were plated as in B. Where indicated, cells were treated with AICAR and/or (PS 300μM) for 45 min. The cells were then harvested and levels of P-AMPK, AMPK, P-ACC, ACC and actin were determined as in B. F, BJ-hTERT cells were plated as in D. Where indicated cells were treated with AICAR and/or (PA 300μM) for 4 hr. The cells were then harvested and levels of P-AMPK, AMPK, P-ACC, ACC and actin were determined as in D. G, MDA-MB-231 cells were plated as in D. Where indicated, cells were treated with AICAR and/or (TPA 100μM) for 24 hr. The cells were then harvested and levels of P-AMPK, AMPK, P-ACC, ACC and actin were determined as in D. Error bars for PLD assays represent S.D. values for at least two independent experiments. The Western blots shown are representative of experiments repeated at least two times. The statistical significance (P value) was determined by a two-tailed, paired Student’s t-test. *P ≤ 0.01 compared with control. Relative levels of AMPK, ACC phosphorylation were normalized to total AMPK, total ACC respectively and quantified as in Fig 4.1A.
4.2.2 Inhibiting AMPK Increases PLD Activity in a Rheb-dependent Manner

The data in Figs. 4.1 indicate that AMPK suppresses PLD activity. We therefore investigated the effect of inhibiting AMPK on PLD activity. We first examined the effect of compound C – a pharmacological inhibitor of AMPK. As shown in Fig. 4.2A, compound C inhibited the phosphorylation of both AMPK and ACC. Compound C also increased the PLD activity in both MDA-MB-231 and Calu-1 cells (Fig. 4.2A). We also suppressed the expression of AMPK and LKB1 using siRNA. As shown in Fig. 4.2B, suppression of either AMPK or LKB1 expression elevated PLD activity in both MDA-MB-231 and Calu-1 cells. Thus, activation of AMPK suppresses PLD activity and suppression of AMPK elevates PLD activity.

AMPK phosphorylates TSC2 and activates its GAP activity towards Rheb (46) – resulting in the suppression of Rheb. Rheb binds to and activates PLD1 in a GTP-dependent manner (56). We therefore examined whether the elevated PLD activity observed in response to suppression of AMPK was dependent on Rheb. We stimulated PLD activity in MDA-MB-231 cells by suppressing AMPK expression with siRNA for AMPK as in Fig. 4.2B and then examined the effect of suppressing Rheb expression on PLD activity. As shown in Fig. 4.2C, suppressing Rheb expression with siRNA prevented the induction of PLD activity observed with AMPK knockdown with siRNA. These data demonstrate that the increased PLD activity observed with AMPK inhibition was dependent upon Rheb.
Figure 4.2 Inhibiting AMPK increases PLD activity in a Rheb-dependent manner. A, MDA-MB-231 cells were plated as in Fig 4.1B, and were then treated with Compound C (Com C) (20μM) for 45 min. The cells were then harvested and levels of P-AMPK, AMPK, P-ACC, ACC and actin were determined by Western blot analysis. For PLD activity assay MDA-MB-231 and Calu-1 cells were plated and treated with
Compound C for 45 min, and the relative PLD activity was then determined as in Fig 4.1C. B and C, MDA-MB-231 cells were plated in 6 well plates at 30% confluence overnight. The cells were then transfected with siRNAs for either scrambled (scram) control siRNA, LKB1 or AMPK (B) or Rheb (C) as indicated. 6 h later, the cells were treated with fresh medium containing 10% serum for an additional 48h. The cells were then harvested and the levels of LKB1, P-AMPK, AMPK (B), Rheb (C) and actin were determined by Western blot analysis. For PLD activity assays, Calu-1 (B) and MDA-MB-231 (B, C) cells were plated and transfected with siRNAs as mentioned above. After 48 h of siRNA transfection cells were harvested and PLD activity was evaluated as in A. Values were normalized to the control scrambled siRNAs, which were given a value of 100%. Error bars for PLD assays represent S.D. values for at least two independent experiments. The Western blots shown are representative of experiments repeated at least two times. The statistical significance (P value) was determined by a two-tailed, paired Student’s t-test. *P ≤ 0.01 compared with control.

4.2.3 Inhibition of PLD Activates AMPK

PLD catalyzes the hydrolysis of phosphatidylcholine to generate choline and PA, which is required for mTOR complex stability (54) and activity (58). Since mTOR is sensitive to the energy status of the cell, we evaluated the effect of suppressing PLD activity on AMPK. We first examined effect of siRNAs for PLD1 and PLD2 on AMPK activity as measured by the level of phosphorylated AMPK and ACC. As shown in Fig 4.3A, introduction of PLD 1 and 2 siRNAs led to the suppression of PLD activity and elevated the level of both AMPK and ACC phosphorylation. Similar results were obtained using catalytically inactive mutants of PLD1 and PLD2 that act as dominant-negative mutants (54) (Fig. 4.3B) or pharmacological inhibitors of PLD1 and PLD2 (Fig. 4.3C). Importantly, the effect of the PLD inhibitors on the phosphorylation of AMPK and ACC could be
suppressed if PA was exogenously provided (Figs. 4.3D and 4.3E) – indicating that the effect of the inhibitors was due to preventing PA production. Thus, there appears to be reciprocal regulation of PLD by AMPK and AMPK by PLD.

Figure 4.3. Inhibition of PLD activates AMPK  A, MDA-MB-231 cells were plated as in Fig 4.2B and were transfected with PLD1 and PLD2 siRNAs or a scrambled control siRNA as indicated. 6 h later, the cells were treated with fresh medium containing 10% serum for an additional 72h. The cells were then harvested and PLD activity was evaluated as described under “Experimental Procedures”. Values were normalized to the control scrambled siRNAs, which were given a value of 100%. For Western blot analysis, cells were harvested at 72 hr and levels of P-AMPK, AMPK, P-ACC, ACC and actin were determined. B, MDA-MB-231 cells were plated as in A and were transfected with vectors expressing catalytically inactive dominant negative (DN) mutant forms of PLD1 and PLD2. The parental vector
pcDNA3.1 was used as control. 48h later, the cells were harvested and levels of P-AMPK, AMPK, P-ACC and ACC were determined as in A. Expression of PLD mutants was evaluated by probing the blots for HA tags on the PLD mutants. Blots were also probed for actin as loading controls. C, MDA-MB-231 cells were plated and the PLDi (PLD1 inhibitor+PLD2 inhibitor, 10μM each) were added for 1 h and the relative PLD activity was then determined as in Fig 4.1C. For Western blot analysis, MDA-MB-231 cells were plated and treated with PLDi for indicated times. Cells were harvested and levels of P-AMPK, AMPK, P-ACC, ACC and actin were determined as in A. D, MDA-MB-231 cells were plated as in C, were treated with PLDi and / or PA for 45 min where indicated. The cells were then harvested and levels of P-AMPK, AMPK, P-ACC, ACC and actin were determined as in A. E, Calu-1 cells were plated and the PLDi (PLD1 inhibitor+PLD2 inhibitor, 10μM each) were added for 1 h and the relative PLD activity was then determined as in Fig 4.1C. For Western blot analysis, Calu-1 cells were plated as in C, were treated with PLDi and / or PA for 45 min where indicated. The cells were then harvested and levels of P-AMPK, AMPK, P-ACC, ACC and actin were determined as in A. The Western blot data are representative of experiments repeated at least two times. Error bars for PLD assays represent S.D. values for at least two independent experiments. The statistical significance (P value) was determined by a two-tailed, paired Student’s t-test. *P < 0.001 compared with control. Relative levels of AMPK, ACC phosphorylation were normalized to total AMPK, total ACC respectively and quantified as in Fig 4.1A.

4.2.4 Activation of AMPK by PLD Inhibition is Due to Reduced mTORC1 Activity— The data presented in Figs. 4.1-3 reveal a reciprocal regulation of AMPK and PLD whereby suppression of AMPK activates PLD, and suppression of PLD activates AMPK. A critical target of PLD and PA is mTOR (71), which like PLD, is dependent upon Rheb (46,132). It was previously reported that rapamycin could increase phosphorylation of AMPK and ACC (136). We therefore examined whether suppression
of mTORC1 would, like suppression of PLD, elevate AMPK activity. We treated MDA-MB-231 cells with siRNAs for both mTOR and the mTORC1 companion protein Raptor and examined the levels of AMPK and ACC phosphorylation. As shown in Fig. 4.4A, suppressing the expression of either mTOR or Raptor led to substantial increases in both AMPK and ACC phosphorylation. We next evaluated the effect of PA on AICAR-induced phosphorylation of TSC and the mTOR substrates S6 kinase and eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1). As expected, AICAR induced phosphorylation of TSC at the AMPK site at S1387 and suppressed phosphorylation of the mTORC1 substrates S6 kinase S6 kinase and 4E-BP1 (Fig. 4.4B, left panel). Importantly, the effect of AICAR was reversed if cells were provided with PA – indicating that the effect of AICAR was the result of suppressing PLD activation. However, if the expression of either mTOR (Fig. 4.4B, middle panel) or Raptor (Fig. 4.4B, right panel) was suppressed with siRNA, PA was unable to reverse the effect of AICAR. These data demonstrate that the presence of PA overcomes the effects of AMPK activation – implicating PLD; and importantly, that suppression of mTORC1 overcomes the ability of PA to suppress the effect of AICAR. The inability of PA to overcome the effect of AICAR in the absence of mTOR or Raptor indicates that the effect of AICAR on mTORC1 was due to the suppression of PLD-stimulated PA production. The inability of PA to rescue the inhibitory effect of AICAR on TSC2 phosphorylation in the absence of either mTOR or Raptor supports the hypothesis that the effect of PA was dependent on mTOR.

We next evaluated the effect of PLD inhibition on autophagy as a readout of mTOR suppression. Autophagy is regulated by several kinases including AMPK, a positive regulator of autophagy(137). AICAR treatment or PLD inhibitors’ treatment induces
phosphorylation of ULK1 Ser555, a specific site mediated by activated AMPK (Fig 4.4C). Contrary to this both treatments also suppresses the phosphorylation of ULK1 Ser757 which is mediated by mTOR. These data suggest that inhibition of PLD results in autophagy by inducing AMPK-ULK1 (Ser555 phosphorylation) and by suppressing mTOR-ULK1 (Ser757 phosphorylation). The effects of AMPK-PLD feedback substantially regulates autophagy through mTOR signaling node. Data provided here reveal that PLD suppresses autophagy through the mTOR-AMPK-ULK1 signaling pathway. These data are consistent with a recent report from Min and colleagues (138), who reported that PLD inhibition enhances autophagic flux via ULK1. These data reveal that activation of AMPK or suppression of PLD contributes to the activation of the autophagic kinase ULK1.
Figure 4.4 Effects of PLD on AMPK are mediated by mTORC1. A, MDA-MB-231 cells were plated as in Fig 3A and were transfected with either mTOR siRNA or Raptor siRNA or a scrambled control siRNA as indicated. After 72h of transfection, cells were harvested and analyzed for P-AMPK, AMPK, P-ACC, ACC, mTOR, Raptor and actin by Western blot analysis. Relative levels of AMPK, ACC phosphorylation were normalized to total AMPK, total ACC respectively and quantified as in Fig 4.1A. B, MDA-MB-231 cells were plated and transfected with mTOR or Raptor siRNA as in A. After 72 hr, cells were treated with AICAR and/or PA as indicated for 45 min. The cells were then harvested and levels of phospho-TSC2 (P-TSC2), TSC2, phospho-S6 kinase (P-S6K), S6K, phospho-4E-BP1 (P-4E-BP1), 4E-BP1 and actin were determined as in A. Relative levels of TSC2, S6K, 4E-BP1 phosphorylation were normalized to total TSC2, S6K and 4E-BP1 respectively and quantified as in Fig 4.1A.
normalized to total TSC2, total S6K, total 4E-BP1 respectively and quantified as in Fig 4.1A. C, MDA-MB-231 cells were plated as in Fig 4.1B, were treated with PLDi or AICAR for 1hr where indicated. The cells were then harvested and levels of P-ULK1, ULK1 and actin were determined as in A. The data shown are representative of experiments repeated at least two times.

4.3. Discussion

The level of nutrients and energy in cells is carefully monitored. Two critical signaling nodes for nutrient and energy sensing are AMPK and mTORC1 with AMPK being activated in response to energy deprivation and mTOR being activated by nutritional sufficiency. An emerging theme in cancer is that these signaling nodes are dysregulated with AMPK being suppressed and mTOR being activated during tumorigenesis (139). One of the more common tumor suppressor genes is LKB1, which contributes to the suppression of AMPK when lost by mutation (140) – underscoring the importance of AMPK suppression in tumorigenesis. In this report, we have provided evidence for reciprocal regulation of AMPK and mTOR involving PLD and its metabolite PA. Suppression of PLD activity increased the phosphorylation of AMPK at the LKB1 site at Thr172 and ACC at the AMPK site at Ser79. Exogenously supplied PA suppressed phosphorylation of AMPK and ACC in an mTORC1-dependent manner.

The suppression of AMPK in response to elevated PLD activity in cancer cells provides a positive feedback loop for sustaining elevated PLD and mTORC1 activity. This keeps the TSC GAP activity suppressed, and thusly, Rheb remains GTP bound and capable of activating PLD1. This is likely the case in cancer cells where PLD activity is commonly elevated (72). However, the reverse could also be true and elevated AMPK
could suppress PLD activity, which would lead to less PA and an inactive mTOR – and as a consequence, prevent the suppression of AMPK. In this case, there is a positive feedback loop that favors AMPK. This is shown schematically in Fig. 4.5. It is not clear at this point what it is that determines which loop will predominate – the one that favors AMPK or the one that favors PLD and mTOR. However, this is an important point with implications for tumorigenesis and possible therapeutic options.

Figure 4.5 Model for Reciprocal regulation of AMPK and PLD. Two scenarios are presented for the regulation of PLD by AMPK and the regulation of AMPK by PLD. A, AMPK is activated by the presence AMP and phosphorylation by LKB1. AMPK then phosphorylates TSC, which stimulates the GAP activity of TSC towards Rheb resulting in the hydrolysis of bound GTP to GDP and inactivation of Rheb. As a consequence, PLD does not get activated (56) and PA needed for stabilization of mTORC1(54) is not generated and mTORC1 is inactive. B, Rheb is GTP bound and promotes the production of PA by PLD and the stabilization of mTORC1. Active mTORC1 then causes suppression of AMPK in a manner that is likely dependent on S6 kinase in that rapamycin has been reported to activate AMPK (136) at concentrations that suppress S6 kinase (50). Other signals promoted by oncogenic stimuli contribute to the activation of this pathway such as mTORC2-Akt, which suppresses TSC (46) and Ras, which leads to PLD activation (141). It is also important to note that mTOR is an integrator on nutrient and growth factor signals.
(25)– both of which are required for cell cycle progression and proliferation. mTOR requires amino acids (142), glucose (55), and possibly lipids (43) for activity.

The regulation of mTOR by AMPK has two separate pathways – one that goes through phosphorylation of TSC2 and activation of the GAP activity towards Rheb (129,130). However, AICAR can still suppress mTOR in TSC2−/− cells, which led to the discovery that AMPK can also suppress mTORC1 by direct phosphorylation of the mTORC1 substrate-recognizing subunit Raptor at Ser792 (143,144). These findings indicate that the relationship between AMPK and mTOR is complex and that there may be multiple connections between the two signaling nodes that monitor nutrient and energy sufficiency. It is not clear how PLD and mTOR suppresses AMPK, however it was reported previously that rapamycin can stimulate the phosphorylation of AMPK at the LKB1 site at Thr172 and ACC at the AMPK site at Ser79 (136). This could be achieved at low nano-molar concentrations of rapamycin that suppress S6 kinase phosphorylation, but have no effect on the phosphorylation of 4E-BP1 (50,145) – suggesting a role for S6 kinase. In contrast with cancer and other proliferating cells, AMPK stimulates glucose uptake when ATP levels are reduced (146). Interestingly, it has been reported that AMPK activates PLD1 in muscle cells and that PLD activation is required for glucose uptake (147,148). This apparently occurs via direct phosphorylation of PLD1 by AMPK at Ser505 and required ERK (147). Whether the PA impacted on mTOR in these studies was not clear, but it has been reported that PLD can stimulate the mTOR-dependent expression of hypoxia-inducible factor1α and 2α (149,150), which promote glucose uptake and glycolysis (151). These studies indicate that AMPK can both suppress and activate PLD1 depending upon metabolic need.
A theme that has emerged in cancer is that it is critical to keep AMPK activity suppressed – as evidenced by the frequency of LKB1 mutations in cancer (77). There are two means for AMPK to suppress mTOR – indirectly through phosphorylation of TSC (129,130), and directly through phosphorylation of Raptor (143,144). It has been speculated that the most commonly dysregulated signals in human cancer are those that lead to elevated mTOR activity (7,152). In this regard, it is significant that PLD activity is elevated in a wide variety of human cancers and cancer cell lines (71,72). The finding here that PLD suppresses AMPK in an mTOR-dependent manner is consistent with a central role for PLD and its metabolite PA in signals involved in the regulation of nutrient and energy status. PA occupies a central spot in membrane phospholipid biosynthesis and is therefore an ideal indicator of lipid sufficiency. It could also indicate sufficient glucose and glutamine, which are key nutrients that are incorporated in to PA (153). We have proposed that the PA requirement of mTOR evolved as a means to sense the presence of sufficient materials for membrane synthesis to complement the ability to sense amino acids (153,154). Consistent with this hypothesis, the enzymes involved in the de novo synthesis of PA stimulate mTOR (155,156) – indicating that the PA requirement of mTOR is related to the ability to synthesis membrane phospholipids.
CHAPTER V

AICAR Enhances the Efficacy of Rapamycin in Human Cancer Cells
5.1. Introduction

In the progression of a normal cell to a cancer cell, it is critical that there be a means to suppress default apoptotic programs that arguably are the first line of defense of cancer (157). A critical signaling node that promotes the survival of cancer cells is mTOR – the mammalian/mechanistic target of rapamycin. There are two mTOR complexes mTORC1 and mTORC2 that have both been implicated in cancer cell survival signals. It has been suggested that the signals that regulate mTOR are the most dysregulated signals in human cancer cells (7). As a consequence, there has been substantial interest in mTOR as a therapeutic target for many human cancers (158,159). Compounds that target mTOR have been employed in many clinical trials (160,161) – albeit without much success. There are distinct classes of compounds that target mTOR: rapamycin and rapamycin analogs (rapalogs) and ATP-competitive inhibitors. Rapamycin is a natural product that acts as an allosteric inhibitor that preferentially inhibits mTORC1 (162). Both classes of inhibitors have inherent problems. The ATP-competitive inhibitors are good in that they target both mTORC1 and mTORC2, which both contribute to survival; however, as with most ATP-competitive inhibitors, there is concern as to specificity for mTOR. In contrast, rapamycin is highly specific for mTOR, but there are peculiar dosage issues associated with rapamycin (162).

Rapamycin inhibits different cells with different dose responses. For example, phosphorylation of the mTORC1 substrate ribosomal subunit S6 kinase (S6K) in MCF7 breast cancer cells is suppressed at 0.5 nM, but in MDA-MB-231 cells, you need 20 nM to suppress S6K (53). This was due at least in part to the levels of phospholipase D (PLD) activity in the two cell lines. PLD generates the metabolite phosphatidic acid (PA),
which interacts with mTOR in a manner that is competitive with rapamycin (53,54,58). Elevating PLD activity in MCF7 cells increased the dose of rapamycin to suppress phosphorylation of S6K, and similarly, reducing PLD activity in MDA-MB-231 cells reduced the dose needed to suppress S6K phosphorylation (53). There is also a problem in that different doses of rapamycin are needed to inhibit the phosphorylation of different mTORC1 substrates. The phosphorylation of S6K can be suppressed by low nano-molar levels; whereas phosphorylation of eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) requires micro-molar doses (50). This is an important issue because the apoptotic effects of rapamycin are due to suppressing phosphorylation of 4E-BP1 (50). The doses that can be achieved in the clinic do not approach the levels needed to inhibit 4E-BP1 phosphorylation (163). This is likely why rapalogs have been largely disappointing in clinical trials in that you cannot deliver doses of rapamycin that overcome the survival effect of mTORC1, which involves primarily the phosphorylation of 4E-BP1 (50).

Another problem with rapamycin is that by suppressing S6K phosphorylation, it suppresses a negative feedback loop that keeps mTORC2 from phosphorylating and activating the survival kinase Akt, and as a consequence, rapamycin activates Akt (60,164). Whereas, the catalytic ATP-competitive inhibitors suppress both mTORC1 and mTORC2 (160), under most conditions, rapamycin suppresses only mTORC1 (50). Thus, activating mTORC2 by rapamycin treatment can lead to elevated Akt activity and suppress the apoptotic effects of rapamycin, which has been observed in pancreatic cancer cells (62).
5.1.1 Purpose of the study in this chapter

In order to take advantage of the high specificity of rapamycin for mTOR, there needs to be a means for making rapamycin effective at lower doses. We reported previously that partial suppression of PLD activity in breast cancer cells resulted in the suppression of Akt at the mTORC2 site at Ser473 with 200 nM rapamycin (54). Thus, suppression of PLD activity can improve the efficacy of rapamycin for both mTORC1 and mTORC2. We very recently reported that PLD activity can be suppressed by activating AMP-dependent protein kinase (AMPK) with the AMP-mimetic compound AICAR (5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside) (165). We therefore investigated the effect of treating cancer cells with the combination of rapamycin and AICAR. In this report we provide evidence that tolerable doses of rapamycin in combination with AICAR suppresses both 4E-BP1 and Akt phosphorylation and induces apoptosis in cancer cells.

5.2 Results

5.2.1 AICAR Treatment Causes S-phase Cell Cycle Arrest

We previously reported that cells arrested in S-phase of the cell cycle could be killed with rapamycin (166). In this regard, it was of interest that activation of AMPK has been shown to cause S-phase cell cycle arrest (167). AMPK can be activated by AICAR – a cell-permeable nucleoside that is metabolically converted by adenosine kinase to 5-aminoimidazole-4-carboxamide ribonucleoside monophosphate or ZMP, which mimics the allosteric effects of AMP and activates AMPK (135). As shown in Fig. 5.1A, there was dramatic increase in cells with S-phase DNA content in both MDA-MB-231 and MCF7 breast cancer cells treated with AICAR as determined by flow cytometry.
suppressed proliferation of the MDA-MB-231 and MCF7 cells – indicating that the cells were actually arresting in S-phase and not just changing the duration of the cell cycle phases (Fig. 5.1B). As shown in Fig. 5.1C, there were decreased levels of cyclin D1, phosphorylated Rb, and proliferating cell nuclear antigen (PCNA); and increased levels of cyclin A2 in both cell lines – consistent with the apparent S-phase cell cycle arrest. These data demonstrate that AICAR induces S-phase cell cycle arrest in these two breast cancer cell lines. Dose responses to AICAR were determined for cell cycle arrest (Fig. 5.1D) and for the phosphorylation of AMPK and the AMPK substrate acetyl-CoA carboxylase (Fig. 5.1E).
Figure 5.1. AICAR treatment causes S-phase cell cycle arrest. A, MDA-MB-231 and MCF-7 cells were plated at 30% confluence in 10-cm plates in DMEM containing 10% serum. After 24 hr, the cells were treated with AICAR (0.5 mM) for 48 hr. After 48 h, the cells were harvested, fixed, stained with propidium iodide, and analyzed for cell cycle distribution by measuring DNA content/cell as described in Methods. The error bars represent the standard error of the mean for experiments repeated three times. B, Cells were plated at 20% confluence in six-well plates in complete media containing 10% serum. After 24 hr, AICAR (0.5mM) was added. Cells were harvested at indicated time points, stained using crystal violet, and quantified by light microscopy as described in Methods. Error bars represent the standard error for an experiment repeated three times. C, Cells were plated at 30% confluence in 10-cm plates in complete media containing 10% serum for 24 h at which time they were treated with AICAR (0.5 mM) for 48 hr. The cells were subsequently harvested and cell lysates were collected. The indicated protein levels were determined by Western blot analysis. The data shown are representative of experiments repeated at least two times. D, Cells were seeded as in B and treated with various concentrations of AICAR (0.25-2mM) for 48 hr, at which time the cells were harvested, stained using crystal violet, and quantified by light microscopy as described in Experimental Procedures. Error bars represent the standard error for an experiment repeated three times. E, Cells were seeded as in C and treated with various concentrations of AICAR (0.25-2mM) for 24 hr. Cells were harvested and the levels of phospho-AMPK, AMPK, phospho-acetyl-CoA carboxylase (P-ACC), ACC, and actin were determined by Western blot analysis.
5.2.2 AICAR Treatment Reduces the Concentration of Rapamycin to Induce Apoptosis

We next treated the MDA-MB-231 cells with rapamycin in combination with AICAR and looked for cleavage of the caspase 3 substrate poly-ADP-ribose polymerase (PARP) as an indicator of apoptosis. As expected based on our previous study (166), arresting cells in S-phase with AICAR resulted in a sharp increase in the level of cleaved PARP when rapamycin was included (Fig. 5.2A). What was not expected was that the dose required for induction of PARP cleavage was 1000-fold lower than that observed previously (50,53,166). PARP cleavage was induced at 20 nM rapamycin; whereas previously, PARP cleavage required 20 μM in MDA-MB-231 cells (Fig. 5.2B). As shown in Fig. 5.2C, the combination of AICAR and 200 nM rapamycin led to increased levels of sub-G1 DNA content in the MDA-MB-231 and MCF7 cells – further supporting an apoptotic cell death. This was also observed in Calu1 lung cancer cells (Fig. 5.2C). Importantly the apoptotic effect was not observed in the non-cancerous BJ-hTERT human fibroblast cell line. We also performed a dose response curve for induction of PARP cleavage by rapamycin on MCF7 cells in the presence of AICAR and as shown in Fig. 5.2D, PARP cleavage could be detected at 0.5 nM. We previously reported that MCF7 cells are much more sensitive to rapamycin than MDA-MB-231 cells and demonstrated that loss of viability in MCF7 cells was observed at 100 nM (53). Thus, like the MDA-MB-231 cells, the presence of AICAR reduced the effective dose of rapamycin needed to induce apoptosis. Consistent with the lack of BJ-hTERT cells containing sub-genomic DNA (Fig. 5.2C), the combination of AICAR and 200 nM rapamycin also failed to induce PARP cleavage in these cells. The data in Fig. 5.2 reveal that AICAR reduces the level
of rapamycin to induce apoptosis in cancer cells, while not inducing apoptosis in the non-cancer BJ-hTERT human fibroblast cell line.

Figure 5.2 AICAR treatment reduces the concentration of rapamycin to induce apoptosis. A, MDA-MB-231 cells were plated at 60% confluence in 60mm plates in DMEM containing 10% serum. 24 hr later the cells were treated with AICAR (2mM) and/or different doses of rapamycin as indicated for 24 hr. The cells were then harvested and levels of cleaved PARP (Cl PARP) and actin were determined as in...
Fig. 5.1C. B, MDA-MB-231 cells were plated as in A. 24 hr later of plating, the cells were shifted to complete medium or medium lacking serum and treated with rapamycin at different doses for 24 hr. The cells were then harvested and indicated protein levels were determined as in A. C, MDA-MB-231, MCF-7, Calu-1 and BJ-hTERT cells were plated at 40% confluence and treated with AICAR (0.5 mM) and/or rapamycin (200 nM) for 48 hr, after which these were collected and subjected to flow cytometric analysis. Total subgenomic DNA is plotted as indicated. Error bars represent S.D. values for at least two independent experiments. D, MCF-7 cells were plated in A. The cells were treated with AICAR (2 mM) and/or varying doses of rapamycin as indicated for 24 hr. The cells were then harvested and indicated protein levels were determined as in A. E, BJ-hTERT cells were plated in A. The cells were treated with AICAR (2 mM) and/or rapamycin (200 nM) for 24 hr. The cells were then harvested and indicated protein levels were determined as in A. The data shown are representative of experiments repeated at least two times.

5.2.3 Apoptotic Effects of AICAR and Rapamycin is Dependent on the Suppression of mTORC2 by Low Dose Rapamycin

We next examined the efficacy of AICAR and rapamycin on mTORC1 and mTORC2 substrates in MDA-MB-231 cells. As shown in Fig. 5.3A, AICAR treatment suppressed the phosphorylation of the mTORC1 substrates S6K and 4E-BP1. However, AICAR stimulated phosphorylation of Akt at the mTORC2 site at Ser473 (Fig. 5.3A). No PARP cleavage was detected with AICAR treatment alone (Fig. 5.3A). Rapamycin (200 nM) suppressed phosphorylation of S6K and weakly suppressed phosphorylation of 4E-BP1 (Fig.5.3A), which is consistent with our previous report that micro-molar concentrations of rapamycin were needed to suppress phosphorylation of 4E-BP1 (50). Rapamycin, by itself, had no effect on the level of Akt phosphorylation or PARP cleavage.
(Fig. 5.3A). The most significant difference between the use of either rapamycin or AICAR by themselves vs rapamycin and AICAR in combination was that rapamycin completely suppressed Akt phosphorylation in the presence of AICAR and induced PARP cleavage (Fig. 5.3A). Thus, AICAR also reduced the concentration of rapamycin needed to inhibit mTORC2 and suppress phosphorylation of Akt at Ser473. The ability of rapamycin to suppress Akt phosphorylation is sufficient to induce PARP cleavage and apoptotic cell death in combination with AICAR, which efficiently suppressed S6K and 4E-BP1 phosphorylation in the absence of rapamycin.

To establish that the suppression of Akt phosphorylation was due to rapamycin, we examined the effect of FK506 on S6K and Akt phosphorylation. Rapamycin inhibits mTOR by combining with FK506-binding protein 12 (FK-BP12) and then binding to mTOR. FK506 also binds FK-BP12 and competes with rapamycin, and thusly has been used to reverse the effects of rapamycin (54). As shown in Fig. 5.3B, FK506 reversed the suppression of both S6K and Akt phosphorylation caused by treatment with both AICAR and 200 nM rapamycin. This result demonstrates that rapamycin is responsible for the suppression of Akt phosphorylation at the mTORC2 site at Ser473.

It was previously reported that under some conditions, rapamycin induces dissociation of mTORC2 components mTOR and Rictor (54,168). We therefore examined whether the combination of AICAR and lower dose rapamycin could dissociate mTOR from Rictor. mTOR was immunoprecipitated from MDA-MB-231 cell lysates and then subjected to Western blot analysis for both Raptor (mTORC1) and Rictor (mTORC2). As shown in Fig. 5.3C, both AICAR and rapamycin, by themselves, cause dissociation of mTOR and Raptor. However, significant dissociation of mTOR from Rictor occurred only
when rapamycin treatment was combined with AICAR (Fig. 5.3C). We also examined the effect of AICAR treatment in MDA-MB-231 cells with knockdown of Rictor, and as shown in Fig. 5.3D, the knockdown of Rictor mimicked the effect of low dose rapamycin with regard to stimulating PARP cleavage and inhibiting Akt phosphorylation. The key results of Fig. 5.3 are summarized in Fig. 5.3E. These data further indicate that the key apoptotic effect of rapamycin in combination with AICAR is suppression of the mTORC2-catalyzed phosphorylation of Akt. Collectively, the data in Fig. 5.3 indicate that the ability of AICAR and rapamycin to kill MDA-MB-231 cells is the result of AICAR suppressing mTORC1 and rapamycin suppressing the feedback activation mTORC2 (60,164).
Figure 5.3. Apoptotic effects of AICAR and rapamycin is dependent on the suppression of mTORC2 by low dose rapamycin. A, MDA-MB-231 cells were plated as in Fig 2A. The cells were treated with AICAR (2 mM) and/or rapamycin (200 nM) for 24 hr. The cells were then harvested and levels of the indicated proteins or phospho-proteins were determined by Western blot analysis. B, MDA-MB-231 cells were plated as in A and were treated with AICAR (2 mM), rapamycin (200 nM), FK-506 (10μM) for 24 h. The cells were then harvested and indicated protein levels were determined by Western blot analysis. C, MDA-MB-231 cells were plated and treated with AICAR (2 mM) and/or rapamycin (200 nM) for 24 hr as in A. At this time, lysates were prepared and subjected to immunoprecipitation with anti-mTOR antibody overnight, and then the mTOR immunoprecipitate (IP:mTOR) was subjected, along with the lysates to Western blot analysis for Rictor or Raptor. Relative protein level of Rictor and Raptor were normalized to mTOR and quantified using LI-COR image studio software. D, MDA-MB-231 cells were plated in 6 well plates at 30% confluence overnight. The cells were then transfected with siRNAs for either scrambled control siRNA, or Rictor as indicated. 6 hr later, the cells were treated with fresh medium containing 10% serum for 48hr. AICAR (2 mM) was then added for an additional 24 hr where indicated. The cells were then harvested and the levels of indicated proteins were determined by Western blot analysis. The data shown are representative of experiments repeated at least two times. E, The key data for Fig. 5.3 are summarized in table form where the most critical numbers are in bold highlighting the key effects of the combination of AICAR and rapamycin.

5.2.4 Effect of AICAR on Rapamycin efficacy is due to suppression of PLD activity

mTOR requires PA for stabilizing both mTORC1 and mTORC2 complexes (54) and for mTOR kinase activity (58). Although there are several sources of PA, the most significant is likely PLD, which catalyzes the hydrolysis of phosphatidylcholine to PA and free choline (169). Importantly, the highly conserved PA-binding domain on mTOR (154)
is at the same site where rapamycin binds (58); and rapamycin binds mTOR in a manner that is competitive with PA (53,54,58). We reported previously that suppressing PA production by PLD reduced the level of rapamycin needed to inhibit both mTORC1 and mTORC2 (53,54). We also reported very recently that activating AMPK with AICAR suppressed PLD activity (165) – suggesting the possibility that AICAR was reducing the dose of rapamycin needed to inhibit mTORC2 by suppressing PLD activity. As reported previously (165), AICAR treatment of MDA-MB-231 cells reduced PLD activity (Fig. 5.4A). To determine whether the reduction in PLD activity was responsible for the increased sensitivity of rapamycin, we added PA to determine whether it would reverse the effect of AICAR on the dose of rapamycin needed to suppress Akt phosphorylation. As shown in Fig. 5.4B, the ability of rapamycin to suppress the AICAR-induced phosphorylation of Akt at Ser473 was reversed by PA. These data are consistent with the effect of AICAR on the rapamycin dose needed to suppress mTORC2 being due to suppressing PLD activity and the production of PA. The dissociation of mTOR from Rictor observed with AICAR and rapamycin in Figure 5.3C, was also reversed with PA. These data are consistent with the effect of AICAR on the rapamycin dose needed to suppress mTORC2 being due to suppressing PLD activity and the production of PA – leading to dissociation of mTOR and Rictor making free mTOR accessible to rapamycin.
Figure 5.4 Effect of AICAR on rapamycin efficacy is due to suppression of PLD activity. A, MDA-MB-231 cells were plated at 70% confluence in 60mm plates. 24 hr later the cells were treated with AICAR (2 mM) for 45 min. \[^{3}\text{H}\]-myristic acid was also added for 4 hr to label lipids. 1-BtOH was added for 20 minutes, and the amount of the PLD-catalyzed transphosphatidylation product, phosphatidylbutanol, was determined as described under “Methods”. Values were normalized to the levels of PLD activity in controls, which were given a value of 100%. Error bars for PLD assays represent S.D. values for at least two independent experiments. The statistical significance (P value) was determined by a two-tailed, paired Student’s \(t\)-test. \(*P \leq 0.01\) compared with control. B, MDA-MB-231 cells were plated as in A and treated with AICAR (2 mM) and/or rapamycin (200 nM) for 8 hr. PA (300μM) was added where indicated. After 8h, the cells were harvested and the levels of the indicated proteins and phosphor-proteins were analyzed by Western blot analysis. For IP experiments, MDA-MB-231 cells were plated and treated with AICAR (2 mM) and/or rapamycin (200 nM) for 24 hr. PA (300μM) was added where indicated. At this time, lysates were prepared and subjected to immunoprecipitation with anti-mTOR antibody overnight, and then the immunoprecipitates were subjected to Western blot analysis for mTOR and Rictor. The data shown are representative of experiments repeated at least two times.

5.3 DISCUSSION

We previously reported that arresting cells in S-phase renders most cancer cells sensitive to the apoptotic effects of rapamycin (166). The ability of rapamycin to induce apoptosis was dependent on the ability to suppress the phosphorylation of the mTORC1 substrate 4E-BP1, which required micro-molar doses of rapamycin (50). A serious problem with rapamycin-based therapeutic strategies is that the micro-molar doses that are required for the apoptotic effect are toxic (163). Another problem is that rapamycin suppresses a negative feedback suppression of Akt phosphorylation by mTORC2 leading
to elevated levels of phosphorylated Akt (60,164) that can overcome the apoptotic effect of rapamycin (62). In this report, we describe a surprising finding that activating AMPK with AICAR not only promotes S-phase arrest, which sensitizes cells to the apoptotic effect of suppressing mTORC1, it also makes mTORC2 sensitive to nano-molar doses of rapamycin that are tolerated in the clinic.

The activation of AMPK by AICAR leads to the phosphorylation of tuberous sclerosis complex (TSC1/2), which acts as a GTPase activating protein for Rheb, and thusly turns off Rheb (170). Rheb is required for the activity of both mTORC1 and PLD1 (170). Of interest, AICAR can suppress the phosphorylation of both S6K and 4E-BP1 with equal efficiency. This is not the case with rapamycin, which inhibits S6K 1000-fold lower doses than it inhibits 4E-BP1 (50). Thus, AICAR can accomplish more than rapamycin accomplishes at conventional nano-molar doses. However, there was an unanticipated benefit of combining AICAR with rapamycin – that being the suppression of PLD activity by AICAR, which sensitizes mTORC2 to rapamycin due to the reduced levels of PA generated (54). This turned out to be critical because, like rapamycin, AICAR suppressed the negative feedback suppression of Akt (60,164), which lead to the phosphorylation and activation of Akt. Activated Akt suppresses the apoptotic effect of suppressing mTORC1 (62). Thus mechanistically, AICAR stimulates S-phase arrest, which sensitizes cells to suppression of mTORC1. However, AICAR also suppresses PLD activity – making mTORC2 sensitive to rapamycin at clinically tolerated doses that can prevent the Akt phosphorylation stimulated by AICAR. Thus, while the combination of AICAR and rapamycin might seem redundant – they both suppress mTORC1 – the ability of AICAR to suppress PLD activity, and as a consequence, make mTORC2
responsive to tolerated doses of rapamycin leads to suppression of mTORC2 as well as mTORC1. Moreover, because AICAR suppresses 4E-BP1 phosphorylation more efficiently than rapamycin, the combination of AICAR and rapamycin leads to better suppression of this mTORC1 substrate that is the most critical for the survival effects of mTORC1 (50). This is shown schematically in Fig. 5.5.

![Figure 5.5 Model](image)

**Figure 5.5 Model**
Model for differential doses of rapamycin needed to suppress mTORC2 in the presence and absence of AICAR. In the upper model, PA levels are high, which strongly favors formation of the highly stable mTORC2 complex. It was proposed that rapamycin inhibits mTORC2 only by binding newly synthesized mTOR before the complex forms. (168) Since mTORC2 is so stable, mTOR complexed with Rictor (mTORC2) effectively never becomes available for binding to rapamycin. However, as indicated in the lower model, when AICAR is present, PLD activity is suppressed and PA levels are reduced. In this case, mTORC2 is destabilized and the equilibrium shifts towards free mTOR, which can bind rapamycin at low doses – and prevent re-assembly.
A key motivation for investigating AICAR in combination with rapamycin was the observation that AICAR arrests cells in S-phase of the cell cycle (167) and that rapamycin kills cells arrested in S-phase (166). That is effectively what was observed with the combination of AICAR and rapamycin. As it turned out AICAR was able to efficiently suppress the phosphorylation of 4E-BP1, effectively negating the need for rapamycin. However, rapamycin, unlike AICAR arrests cells in G1 (166), indicating that activating AMPK does more than suppress mTORC1 in causing S-phase rather than G1 arrest. And a critical factor for the apoptotic effect of mTORC1 suppression is arresting the cells in S-phase (166). Ironically, the critical contribution of rapamycin to the apoptotic effect of rapamycin in combination is the suppression of mTORC2-mediated activation of Akt.

With regard to the importance of targeting mTORC2 and the phosphorylation of Akt, ATP-competitive catalytic mTOR kinase inhibitors have been developed that target both mTORC1 and mTORC2 and can suppress the phosphorylation of S6K, 4E-BP1 and Akt at Ser 473. (171) Thus, in principle, the catalytic inhibitors are ideal therapeutic agents for treating cancers where the activation of Akt by suppression of mTORC1 is preventing apoptosis. Consistent with this idea, we have found that torin1 kills BxPC3 pancreatic and that AZD8055 kills MCF7 breast cancer cells where Akt phosphorylation is elevated in response to rapamycin treatment. (50,172) However, we have also observed that Torin1 arrests MDA-MB-231 cells under conditions where rapamycin induces apoptosis (our unpublished observations). Thus, conditions exist where rapamycin is more toxic than a catalytic inhibitor. In addition, several adverse effects have been noted for the catalytic inhibitors due to off-target effects of ATP analogues as well as potential additional effects of suppressing mTORC2. (173) Therefore, the high specificity of
rapamycin for mTOR – even at the high micro-molar doses (50) – still has some advantages over the catalytic inhibitors. The impact of AICAR on the dose of rapamycin needed reported here further enhances the relevance of rapamycin as a therapeutic anti-cancer agent.

The findings reported here have potential clinically important implications. Activators of AMPK have been in use for many years to treat type II diabetes (78). Rapamycin and rapalogs have been widely employed in clinical trials (161). Thus, targeting both AMPK and rapamycin has been widely employed in the clinic suggesting the feasibility using AMPK activators in combination with rapalogs. This combination has previously been reported to be effective in suppressing the proliferation of acute lymphoblastic leukemia cells (174). In addition, a phase I study with the rapalog temsirolimus in combination with metformin in advanced solid tumors has been performed with some positive responses (175). Although no dramatic responses were reported, the study was restricted to limited set of solid tumors.
CHAPTER VI

Conclusions and Future Directions
In chapter 3 we have concluded that K-Ras-driven cancer cells have been resistant to therapeutic intervention and K-Ras itself has been considered undruggable (176). Thus, alternative strategies are needed for what may be as many of 30% of human cancers that are driven by K-Ras mutations(117). The observation that K-Ras-driven cancer cells override a late G₁ Gln-dependent cell cycle checkpoint and arrest in a part of the cell cycle where they are sensitive to cytotoxic drugs represents a potentially exploitable vulnerability of K-Ras-driven cancer cells. The ability to target Gln utilization in combination with cell cycle phase specific cytotoxic drugs could provide a means for targeting a significant percentage of human cancers that have been resistant to therapeutic intervention.

The most interesting and clinically significant future direction will be to confirm these studies in mouse model. KRAS mutant cells may fail to execute the nutrient-sensing checkpoint during glutamine starvation and this might have an impact on the later phases of cell cycle. Glutamine is an important nitrogen donor for nitrogenous bases for DNA synthesis and amino acids for protein synthesis. So when cells proceed to S phase, DNA replication may be slowed down due to shortage of nucleotides. Similarly, protein synthesis could be slowed down during G2 phase due to shortage of amino acids and this might lead to aberrant M phase. The prolonged S phase and G2/M phase might provide a broader time-window for the actions of cytotoxic drugs. Additional experiments could be carried out to check for these possibilities. Here are some examples of future experiments: a. Synchronizing cells (e.g., by flow cytometer) and grow them in glutamine deprived medium, monitor their cell cycle progression in a time course. This way, length
of each phase could be estimated. b. Examine Chk1 and Chk2 phosphorylation in the presence/absence of glutamine, with or without capecitabine. It will be also interesting to see whether pharmacological inhibitor of GLS (BEPTES) or genetic knockdown of GLS complement any results that stem from a blockade from glutaminolysis.

In chapter 4 our data indicate that the elevated PLD activity commonly observed in human cancer cells (71,72) contributes to the suppression of AMPK via activation of mTOR. In this study we have provided evidence for a reciprocal regulation of PLD and mTOR by AMPK; and the regulation of AMPK by PLD and mTOR. Given that AMPK is an important mediator of energy homeostasis, it is not surprising that AMPK regulates the level of PA – a metabolite at the center of membrane lipid biosynthesis and a critical factor for mTOR activity. In future study it will be interesting to show Rheb-independent activation and lysosomal translocation of mTORC1 is dependent on PLD activity.

In chapter 5 the study did reveal that the combination of AMPK activation and rapalog is well tolerated. It will be important to establish whether there is a subset of cancers with specific genetic alterations that are especially sensitive to this two-pronged therapeutic approach. The clinically significant future direction will be to confirm these studies in mouse model.
CHAPTER VII

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