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Lipid dependence in Ras-driven tumors

Darin Salloum
Graduate Center, City University of New York

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LIPID DEPENDENCE IN Ras-DRIVEN CANCER CELLS

By Darin Salloum

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

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Date                     Chair of the Examining Committee
Dr. David A. Foster

Date                     Executive Officer
Dr. Laurel A. Eckhardt

Dr. Laurel Eckhardt, Hunter College

Dr. Mitchell Goldfarb, Hunter College

Dr. Gilbert Di Paolo, Columbia University

Dr. Anant K. Menon, Weill Cornell Medical College

Supervisory Committee

The City University of New York
Abstract

MUTANT Ras ELEVATES DEPENDENCE ON SERUM LIPIDS AND CREATES A SYNTHETIC LETHALITY FOR RAPAMYCIN

By Darin Salloum

Adviser: Professor David A. Foster

Over past decade, metabolic alterations in cancer cells have received a substantial amount of interest. It had been established that cancer cells undergo a significant amount of metabolic alterations, and some of these alterations are similar to those in normal highly proliferative cells. However, it is becoming more apparent that many of the metabolic alterations are specific to particular oncogenic signaling pathways. Although altered metabolic machinery makes cancer cells more efficient at promoting growth when nutrients are supplied at the sufficient amounts, the dependency of cancer cells on particular metabolic reprogramming deems cancer cells susceptible to disruptions within metabolic network. Thus, the identification of metabolic weaknesses of cancer cells create a platform for therapeutic interventions.

The conversion of normal cells to cancer cells involves a shift from catabolic to anabolic metabolism involving increased glucose uptake and the diversion of glycolytic intermediates into nucleotides, amino acids and lipids needed for cell growth. An underappreciated aspect of nutrient uptake is the utilization of serum lipids. We investigated the dependence of human cancer cells on serum lipids and report here that Ras-driven human cancer cells are uniquely dependent on serum lipids for both proliferation and survival. Moreover, Ras-driven cancer cells fail to adapt lipid metabolism upon lipid deprivation. Removal of serum lipids also sensitizes
Ras-driven cancer cells to rapamycin. Suppressing pinocytosis in Ras-driven cancer cells similarly created sensitivity to suppression of mTORC1 - the mammalian/mechanistic target of rapamycin.

The findings reported here reveal an enhanced need for serum lipids in Ras-driven human cancer cells that creates a synthetic lethal phenotype for suppressing mTORC1. While depriving humans of serum lipids is not practical, suppressing uptake of lipids is possible and could be exploited therapeutically, presenting speculated that this property displayed by Ras-driven cancer cells represents an Achilles’ heel for the large number of human cancers that are driven by activating Ras mutations.

mTOR has long been known to respond to amino acids, glucose and energy. However, lipids are another essential nutrient, and sensing mechanism on sufficiency of lipid precursors is not yet known. Based on the central position of PA in lipid biosynthesis, and its involvement in mTOR regulation, here we show that PA feeds into mTOR as a metabolite for sensing lipid precursors. In Ras-driven cells, PLD activity increase due to lipid withdrawal may be a mechanism to keep mTOR active during metabolic insufficiency stress.
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List of abbreviations

4E-BP1: eIF4E-binding protein 1
ACC: acetyl-CoA carboxylase
acetyl-CoA: acetyl-coenzyme A
ACLY: adenosine triphosphate (ATP)-citrate lyase
CL: cardiolipin
DG: diacylglycerol
DGK: diacylglycerol kinase
DHAP: dihydroxyacetone phosphate
FA: fatty acid
FAT domain: FRAP-ATM-TRAAP domain
FKBP12: FK506-binding protein 12
G3P: glycerol 3-phosphate
HEAT domain: Huntingtin, elongation factor 3, alpha-regulatory subunit of protein phosphatase 2A and TOR1 domain
LPAAT: lysophosphatidic acid acyltransferase
mLST8: mammalian lethal with sec-13 protein 8
mSin1: mammalian stress-activated map kinase-interacting protein 1
mTOR: mechanistic Target of Rapamycin
mTORC1: TOR complex 1
mTORC2: mTOR complex 2
PA: phosphatidic acid
PG: phosphatidylglycerol
PI: phosphatidylinositol
PI-3K: phosphatidylinositol 3-kinase
PLC: phospholipase C
PLD: phospholipase D
PRAS40: proline-rich Akt substrate 40 kDa
protor1/2: protein observed with rictor 1 and 2
raptor: regulatory-associated protein of mammalian target of rapamycin
Rictor: rapamycin-insensitive companion of mTOR
S6K1: S6 kinase 1
SCD: stearoyl-CoA desaturase-1
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CHAPTER I
INTRODUCTION
1.1. CANCER. HALLMARKS OF CANCER AND METABOLIC TRANSFORMATION

1.1.1 SIX HALLMARKS OF CANCER. AN EMERGING HALLMARK

Cancer, a disorder first written record of which appears in 3000BC [1] and known medically as malignant neoplasm, is a broad number of diseases that involves unregulated cell growth [2, 3]. In order for a cell to reach cancerous state, six biological capabilities must be acquired, described in Weinberg’s review, Hallmarks of Cancer [4]. These include ensuring sustained proliferative signaling, or growth factor independence, circumventing actions of growth factor suppressors, evasion of cell-to-cell “contact inhibition” and activation of extravasation, leading to malignant invasiveness, resisting cell death, enabling replicative immortality and inducing angiogenesis. An emerging attribute of cancer phenotype also involves a major reprogramming and rewiring of cellular metabolic machinery for sustained cell growth and proliferation [5-9]. Not surprisingly, major metabolic sensing machinery, mammalian/mechanistic Target of Rapamycin (mTOR), is commonly upregulated in cancers [10-12]. Here, I will provide evidence for an additional role of mTOR as a metabolic sensor of lipid sufficiency that, when deregulated, contributes to cancer cell survival.
1.1.2 METABOLIC REWIRING. WARBURG AND BEYOND

Cancer cells undergo significant metabolic rewiring and reprogramming that shifts metabolic state from catabolic to anabolic state to sustain continuous growth and proliferation [13, 14]. Otto Warburg was first to observe that cancer cells display altered metabolic regulation: despite having sufficient amount of oxygen for oxidative phosphorylation, cancer cells undergo energetically less efficient glycolysis and produce increased amount of lactic acid, the process that is famously known as Warburg effect, or aerobic glycolysis [15, 16]. Although the observation of the shift to aerobic glycolysis was made more than 80 years ago, the underlying mechanism of the shift is not well elucidated and is under close scrutiny [17, 18]. Importantly, it is not yet clear whether alterations in cellular metabolism are contributors or mere consequence of increased cellular division rate that is observed in normal proliferating tissue [19]. However, metabolic shift in cancer cells in not limited to the Warburg phenomenon, as not all cancer cells display enhanced uptake of glucose [20]. Nevertheless, it has become well recognized in the past decade that a metabolic shift is a necessary occurrence in tumor development and establishment to meet increased energetic and anabolic needs of highly proliferative tissue [21, 22]. Moreover, it had been established that deregulation of many canonical signaling pathways in tumors lead to alterations in metabolic machinery, and mutations that lead to deregulation of cellular metabolism are being studied more extensively [5, 7, 9]. A schematic representation of six hallmarks of cancer with additional proposed hallmarks and their relation to known metabolic reprogramming is shown in Figure 1.
1.1.3 METABOLIC REWIRING. ENERGETIC NEEDS OF DIVIDING CELLS

In order for a cell to produce two viable daughter cells, it must replicate all of its cellular contents. That would include doubling in nucleic acid content for DNA synthesis, lipid mass, proteins and sufficient energy to proceed with mitosis. Thus, cell division imposes large...
requirement for exogenously or endogenously derived macromolecules: ATP, nucleic and amino acids and lipids. In addition, the cell must ensure that it could properly sense and determine sufficiency of macromolecule precursors to proceed with cell division and produce two viable cells.

During cell cycle progression, cells in G1 cell cycle commit to dividing via two distinct and independent restriction points [23, 24]. Firstly, the cell receives instruction to divide in form of growth factors. As mentioned earlier, growth factor dependence in cancer cells is often circumvented via various means [4]. Secondly, the cell must ensure that there are sufficient resources to proceed with cell division. Increased biosynthesis of macromolecules through alternative means is under close research, and much has been learned on how nucleic acids and amino acid levels are maintained in cancer cells [25-28]. It is now evident that in addition to glucose, cancer cells utilize glutamine as a nitrogen source for nucleotides and as a carbon source [29]. Cancer cells also need essential amino acids that mammalian cells cannot synthesize. However, an underappreciated aspect of nutrient uptake is the utilization of exogenously supplied fatty acids [30]. Cells grown in culture are provided with media that is supplemented with glucose, essential amino acids, and glutamine as nutrients for cell growth. However, mammalian cell do not synthesize all of the unsaturated lipids needed for membrane biosynthesis – there are “essential fatty acids” that must also be present in the medium [30]. Conventional growth media used for culturing mammalian cells do not contain lipids – they are provided in the serum that typically supplement culture media.

Thirdly, a nutrient sufficiency sensing mechanism to proceed with mitosis is needed. mTOR, which will be described in more detail in a later sector, is a major sensor of amino acid,
glucose, energy and stress factors [31, 32]. Although much is known how mTOR serves as a responder to nutrients, it is not clear how cells sense presence of sufficient lipid precursors. In this work, I will propose that mTOR is indeed a suitable candidate as a sensor of lipid sufficiency and show that improper lipid sufficiency sensing represents an Achilles’ heel in a population of Ras-driven cancers.

1.2 MAMMALIAN TARGET OF RAPAMYCIN (mTOR)

1.2.1 mTOR OVERVIEW

Mammalian (or mechanistic) Target of Rapamycin functions as an integrator of nutritional, energy and stress status, and its output dictates most major cellular functions that regulate cell growth and proliferation. mTOR is an atypical serine/threonine protein kinase that belongs to phosphatidylinositol 3-kinase (PI-3K) related protein kinase family that is conserved from yeast to mammals [33]. It received its name from a small molecule rapamycin, which is a macrocyclic lactone produced by Streptomyces Hygroscopicus bacteria. Rapamycin has received broad attention in clinic because of its antiproliferative and immunosuppressant effects. Rapamycin forms a complex with the peptidyl-prolyl cis-trans isomerase FKBP12 (FK506-binding protein) and the FKBP12-rapamycin complex binds to mTOR and works as an allosteric inhibitor of the mTOR kinase [34-36]. mTOR forms a catalytic core of at least two functionally and structurally distinct complexes- TOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). These
complexes contain shared and unique partners, display differential sensitivity to rapamycin and have different upstream inputs and downstream outputs [37, 38].

The nomenclature of mTOR has been a subject of a recent debate and, more importantly, confusion [39]. TOR has been originally discovered in yeast by Michael Hall’s group [40, 41]. As mammalian Target of Rapamycin (mTOR), it has been known for almost two decades since the discovery that yeast TOR ortholog exists in mammals [42-45]. However, recently, an alternative name, Mechanistic Target of Rapamycin (MTOR), was introduced by database curators. The interchangeability of the mTOR/MTOR, mammalian/mechanistic, is not widely accepted, and is bringing unnecessary confusion to the field. For the simplicity reasons, and since it is more appropriate from biological point perspective, here I will use the name of mTOR as mammalian Target of Rapamycin.

1.2.2. mTOR STRUCTURE

mTOR is a 290 kDa multi domain protein that belongs to PI-3K related kinases. Members of this kinase family are characterized by presence of N-terminal HEAT (Huntingtin, elongation factor 3, alpha-regulatory subunit of protein phosphatase 2A and TOR1) domain and a kinase domain in the C-terminal half that is flanked by the FAT (FRAP-ATM-TRAAP) domain and FATC domain. HEAT and FAT domains are involved in protein-protein interactions, while FATC domain responds to redox potential [36, 46, 47]. mTOR also possesses self-descriptive FKBP12/rapamycin binding (FRB) domain lying between FAT and kinase domains. Schematic of the structure is shown in the Figure 2.
1.2.3 mTOR COMPLEX COMPOSITION

mTOR complexes contain shared and unique partners. Each complex contains catalytic mTOR subunit, mammalian lethal with sec-13 protein 8 (mLST8), critical for mTOR assembly and signaling, DEP domain containing mTOR-interacting protein (DEPTOR), a negative regulator of mTOR, and the Tti1/Tel2 complex, which regulates stability and assembly of mTOR. mTORC1 contains two unique partners: regulatory-associated protein of mammalian target of rapamycin (raptor), a scaffolding protein that regulates substrate binding, and proline-rich Akt substrate 40 kDa (PRAS40), a negative regulator of mTORC1. mTORC2 contains the following three unique partners: rapamycin-insensitive companion of mTOR (rictor), a scaffolding protein that regulates substrate binding, mammalian stress-activated map kinase-interacting protein 1 (mSin1), and protein observed with rictor 1 and 2 (protor1/2), both of which are involved in binding and activation of mTORC2 downstream target SGK1 [37, 38].
addition, both complexes contain a lipid metabolite, phosphatidic acid (PA), that has been shown to be required for the stability and function of the complexes and binds to mTOR in a manner that is competitive with rapamycin [48-52]. Figure 3 shows the list of components with their known functions.

![Figure 3. mTORC1 and mTORC2 components and their known functions (adapted from (37) with modifications). mTORC1 and mTORC2 components are listed. Unique partners of mTORC1 are coded in blue, unique mTORC2 partners are designated in orange. Shared partners are coded in purple.](image)

1.2.4. mTOR SIGNALING

In essence, mTOR could be viewed as an integrator of diverse cellular cues, and the output dictates major steps for cell growth and proliferation. The substrate composition of the mTOR complexes dictates the substrate specificity. Little is known on upstream regulation of mTORC2, but it is generally agreed that growth factors control mTORC2. mTORC1, regulation of which is better understood, integrates growth factor, amino acid, energy and stress. In addition, both
mTOR complexes have been shown to be regulated by lipid metabolite, PA. Although it has been shown that PA is necessary for mTOR complex formation and stability, the underlying involvement of PA in the function of mTOR is not widely accepted.

mTORC1 exerts much of it function via two substrates, S6 kinase 1 (S6K1) and eIF4E-binding protein 1 (4E-BP1), both of which associate with a particular population of mRNAs and regulate mRNA translation initiation and progression [53]. mTOR-dependent regulation of translation leads to cell growth and proliferation, inhibition of autophagy, macromolecule synthesis, including nucleic acids and lipids [27, 54, 55]. Thus, mTOR regulates metabolic programs to promote cell growth. Downstream targets of mTORC2, Akt, SGK1 and PKCα are essential for promoting cell survival and proliferation as well as regulation of actin cytoskeleton. Schematic of mTOR complexes upstream inputs and downstream outputs is shown in Figure 4.

Figure 4. mTORC upstream input and downstream output (adapted from (37) with modifications). mTORC1 responds to amino acids, stress, oxygen, energy, and growth factors and is acutely sensitive to rapamycin. It promotes cell growth by inducing and inhibiting anabolic and catabolic processes, respectively, and also drives cell-cycle progression. mTORC2 responds to growth factors and regulates cell survival and metabolism, as well as the cytoskeleton.
We have additionally proposed and additional function of mTOR as a sensor of lipid sufficiency [56] that is exerted through PA.

1.3 PHOSPHOLIPASE D (PLD) AND PHOSPHATIDIC ACID (PA)

1.3.1 PLD AND PA OVERVIEW

Phospholipase D is an evolutionarily conserved from bacteria to mammals lipase that catalyses the hydrolysis of the membrane phospholipid phosphatidylcholine to generate choline and the signaling lipid PA. There are two isoforms of PLD (PLD1 and PLD2) that exist in mammals that share about 50% of sequence homology. The two mammalian isoforms are regulated differently, with PLD2 being active at basal level while PLD1 being responsive to growth factors and nutritional stimuli [57-61].

PLD has been implicated in a number of cellular activities, including membrane trafficking, cytoskeletal reorganization and cell migration, exocytosis and receptor endocytosis [62-64]. 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 [52, 65, 66].

PA itself 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, thus, it stands in the center of lipid synthesis, both for membrane phospholipids and triglyceride synthesis [56, 67]. The features of PA as a signaling
molecule and as a central metabolite in lipid synthesis place PA in unique position as a sensing molecule that would be discussed in later section.

1.3.2 MANY SOURCES AND DESTINATIONS OF PA

PA is a unique lipid that serves as both signaling molecule and an essential precursor for membrane phospholipid and triglyceride synthesis. Schematic representation of sources and destinations of PA in lipid metabolism are shown in the Figure 5. Generation of PA for lipid synthesis occurs via three distinct mechanisms. The first mechanism involves hydrolysis of phosphatidylcholine by PLD to produce PA and free choline. However, it is unlikely that PLD produced PA goes into generation of the membrane phospholipid considering that the original source is the membrane phospholipid. The second mechanism involves both newly synthesized and dietary fatty acids. PA is produced by enzymatic reaction of acylation of glycerol 3-phosphate (G3P) and two fatty acids (FAs) by two distinct acyltransferases, the last step of which is catalyzed by lysophosphatidic acid acyltransferase (LPAAT). Fatty acids for this process could be derived from either diet or from newly synthesized FA’s through action of fatty acid synthase (FAS). Importantly, this mode of PA generation involved a glycolytic shunt, as G3P is converted from dihydroxyacetone phosphate (DHAP), glycolytic pathway intermediate. The third pathway involves phosphorylation of diacylglycerol (DG) by DG kinase. DG comes from either stored triglycerides that are released by actions of lipase, or in response to growth factor induced stimulation of phospholipase C (PLC) from phosphatidylinositol.
Destinations of PA for lipid synthesis are multiple. PA could be converted back to DG via action of PA-phosphatase. DG could be acetylated to generate triglycerides for fat storage. PA could also be condensed with cytidine triphosphate (CTP) via action of CDP-diacylglycerol synthase and utilized immediately for the synthesis of phosphatidylglycerol (PG), and cardiolipin (CL), and of phosphatidylinositol (PI) [56, 67] for membrane biosynthesis.

Thus, PA stands at the center of the lipid synthesis. Major metabolic enzymes that catalyze conversion of PA, LPAAT, PLD and DGK, also regulate the passage of PA into storage mode (triglyceride synthesis) or synthetic mode (membrane phospholipids). Given the central location of PA in lipid metabolism, and its input into mTOR, we have speculated that PA requirement of mTOR has evolved as a responsiveness of mTOR to the presence of sufficient lipid precursors.
Figure 5. Phosphatidic acid metabolism (adapted from (55)). PA could be generated via three different pathways. First involves PLD hydrolysis of phosphatidylcholine. Second pathway involves acylation of G3P by glycerol-3-phosphate acyltransferase and lysophosphatidic acid acyltransferase. Third mechanism occurs via phosphorylation of DG by DGK that come as a product of hydrolysis of triglycerides. PA could be later converted into storage lipids, through action of PA phosphatases and then acyl transferase, or membrane lipids, through condensation of PA to CTP to produce CDP-diacylglycerol that is later utilized for glycerophospholipid synthesis.
1.4 mTOR REGULATION BY PLD AND PA

1.4.1 mTOR and PA

PA has been shown to be a critical activator of mTOR signaling. It has been shown that PA binds to mTOR in a manner that is competitive with rapamycin [52], and PA-mTOR interaction is required for the stability of the complexes [50, 51]. In vitro, PA physically associates with the FRB domain of mTOR, and this lipid-protein interaction is highly specific for PA [48]. In addition, the PA-FRB domain association is disrupted by the FKBP12-rapamycin, indicating competitive manner of the binding between PA and FKBP12-rapamycin to mTOR. Solution of the PA-FRB complex structure using NMR spectroscopy also confirmed mutually exclusive mode of FRB binding to PA and/or FKBP12-rapamycin [68]. It also showed a critical role of amino acid residue at the FRB domain, Arg2109, for PA binding. Of a note, the site of PA-FRB domain interaction is highly conserved from yeast to humans, with slight variation of positively charged amino acids, Arg or Lys, at the site [56]. Schematic representation of PA-binding domain of mTOR is shown in Figure 6. It is somewhat evident that the strict conservation of Arg2109 at the FRB domain of mTOR was maintained not for the purpose of creating sensitivity for rapamycin.

There are multiple sources of PA in the cell that contribute to activation of mTOR. PLD is an obvious source, and overexpression of either PLD1 or PLD2 activates mTORC1 in various cells[50]. However, recent reports have showed that whole organism knockout of both PLD1 and PLD2 is viable [69, 70], while mTOR knockouts are embryonic lethal[71, 72], indicating that PA must be used from different sources. Two additional PA-producing enzymes, lysophosphatidic
acid acyltransferase (LPAAT) and diacylglycerol kinase (DGK), have also been shown to regulate mTOR signaling through PA [73, 74].

Thus, a lot of evidence indicates PA involvement in mTOR regulation that go beyond mTOR modulation by PLD. However, the relevant cause for the downstream PA-mTOR signaling remains elusive. It is clear that PA stands at the center of lipid metabolism. In addition, it serves as a signaling molecule to activate major metabolic sensing machinery in the cell, mTOR. Taken these information together, it had been proposed that PA feeds into mTOR as an indicator of lipid sufficiency in the cell [56].

Figure 6. Conservation of PA-binding domain of mTOR (adapted from (55)). The PA-binding domain of mTOR is within the region of mTOR that also binds rapamycin. The sequence contains a critical Arg residue at position 2109 that is critical for PA binding. There is also a conserved positively charged amino acid at the adjacent position 2110, as well as the region flanking these two positive charges.
1.4.2 mTOR, PLD, PA AND HUMAN CANCERS. METABOLIC SENSING

PLD could be in essence viewed as an oncogene. Its activity had been shown to be upregulated in numerous cancers, contributing to survival signals and transformation [66, 75]. PLD activity is also strongly increased in Ras-driven cancer cells, and PLD1 activity has been shown to be required for Ras-induced transformation [76, 77].

mTOR has key roles in regulation of many anabolic and catabolic pathways that are involved in cell proliferation and survival, and it is not a surprise that upstream signals that regulate mTOR are the most commonly dysregulated in cancer [10, 11]. In addition, there are reports of gain-of-function mutations in mTOR protein itself, though these are less common [78]. mTOR significantly contributes to metabolic reprogramming in cells. Increase in ribosome biogenesis linked to mTOR activation probably promotes cell proliferation by providing the machinery required to sustain high levels of cell growth. Dysregulation of cap-dependent translation that is regulated by mTORC1 promotes cell-cycle progression and cell proliferation [79]. Increased de novo lipid biosynthesis is an additional absolute requirement for proliferating cancer cells to support synthesis of new membranes [80]. mTORC1 has been shown to be required for the activation of the prolipogenic factor SREBP1, a master regulator of lipo- and sterolgenic gene transcription [81]. Thus, mTOR not only responds to nutrients and energy, but also contributes to metabolic reprogramming to promote cell survival and proliferation.

PLD has been known to respond to mitogenic stimuli, and subsequently feed on mTOR for quite some time [48, 82]. The regulatory role of PLD on mTOR is also extended as a sensor of
amino acid sufficiency [60, 61, 83]. However, it is becoming more evident that regulation of mTOR by PA is accomplished not only from the hydrolysis of phosphatidylcholine by PA, but also through PA generated from de novo synthesis pathways (reviewed in [56]). Thus, regulation of mTOR by PA could be extended beyond its regulation by PLD.

Importantly, both LPAAT and DG kinase have been shown to stimulate mTOR [73, 74]. Moreover, suppression of LPAAT, which is responsible for de novo synthesized and dietary lipids, suppressed mTOR activity and disrupted survival and proliferative signals in several cancer cell lines [84]. Thus, there is a link between mTOR activation and de novo lipid synthesis through PA. PA hence is proposed to serve as a metabolic sensor to mTOR to corroborate sufficiency of lipid precursors in proliferating cell [56].

1.4.3 DIFFERENTIAL REGULATION OF mTOR BY PA

Most mammalian cells acquire lipids from the diet, however, actively dividing cells have absolutely depend on de novo lipid synthesis [85, 86]. De novo lipid synthesis is largely catalyzed by fatty acid synthase (FASN), and levels of FASN are expressed in high level in many cancers and linked to poor outcome [87-89]. The acetyl groups for fatty acid synthesis come from citrate, using either glucose or glutamine as an anaplerotic carbon source for TCA cycle intermediates [90, 91]. Citrate is converted into acetyl-coenzyme A (acetyl-CoA) by adenosine triphosphate (ATP)-citrate lyase (ACLY), and acetyl-Co A is converted to malonyl-CoA by acetyl-CoA carboxylase (ACC). Subsequent steps of fatty acid synthesis are accomplished by FASN, and the final generated product is palmitic acid, a basic 16-carbon saturated fatty acid [92]. Palmitate could be elongated to stearate and then desaturated by stearoyl-CoA desaturase-1 (SCD1) to form monounsaturated oleic acid.
The generated FA could be incorporated into membrane biosynthesis with the help of acyltransferases. However, the lipid species that come from de novo synthesis are either completely saturated, or have one desaturated group. Hence, mammalian cells require exogenously supplied unsaturated fatty acids, essential fatty acids, such as linoleic and linolenic, which contain 2 and 3 double bonds respectively [93, 94].

Recent reports had shown that there is differential activation of mTOR by completely saturated vs. partially unsaturated PA species. PA species with two saturated palmitates had been shown to inhibit mTORC2 [95], while 1-palmitoyl, 2-oleoyl-PA is stimulatory for both mTORC1 and mTORC2 [51, 96]. Thus, some degree of desaturation on PA is needed for

![Diagram](image_url)

**Figure 7. Regulation of mTOR by PA species (adapted from (55)).** Two major destinations for newly synthesized PA are either into membranes biosynthesis when cells are in proliferative mode, or into synthesis of triglyceride, when cells are in storage mode. Both PA species interact with mTOR. Di-unsaturated PA interaction with mTOR leads to mTOR inhibition, while interaction of at least partially unsaturated PA with mTOR leads to mTOR activation.
activation of mTOR. Alternatively, it is possible that only PA species with some degree of desaturation lead to stabilization of the complexes, while completely saturated PA species binding leads to dissociation of mTOR complexes. Schematic of mechanistic sensing of PA species by mTOR is shown in the Figure 7. PA exerts its regulatory function on mTOR by activating mTOR with PA some level of desaturation, and thus shifting metabolic machinery to produce membrane phospholipids. Alternatively, PA species with two saturated FAs inhibit mTOR, and the majority of saturated PA is shunted into storage mode.

1.5 RAS IN CANCERS. RAS-MEDIATED ACTIVATION OF PLD

Ras GTPases (H, K, N-Ras) are the key proteins in eukaryotic signal transduction directed toward cellular proliferation and differentiation, and it regulates a myriad of pathways in the cell. The biological activity of Ras is regulated by GDP/GTP loading cycle. Guanine nucleotide exchange factors (Ras-GRF, Sos) induce dissociation of GDP from Ras-GDP to form an active, GTP-bound form of Ras. GTP-ase activating proteins (NF1, p120GAP) accelerate the intrinsic GTP hydrolytic activity of Ras to promote the formation of an inactive GDP-Ras [97-99]. Not surprisingly, one of the most common gain of function mutations found in human cancers are activating mutations to genes encoding Ras family GTPases [100], with 30% of all cancers and 90% of all pancreatic cancers carrying Ras mutation that are thought to be driver mutations [101].

Many cancer cell lines with elevated PLD activity have activating mutations in Ras [77]. In addition, activated Ras had been shown to stimulate increases in PLD activity. Moreover, PLD1 isoform is constitutively associated with RalA[102], a downstream target of Ras [103]. Thus, a critical target of Ras signaling in cancer pro-survival could be PLD.
1.6 PROJECT RATIONALE

Cancer cells have been shown to be efficient in ignoring many signals to which normal cells respond. Metabolic signals are no exception. It is proposed that cancer cells rewire and trick metabolic sensing into “thinking” that there is sufficient amount of lipid precursors. The PA species, most commonly associated with mTOR regulation, is generated by the hydrolysis of phosphatidylcholine by PLD. However, reports have revealed that dual PLD1 and PLD2 knockouts are viable [69, 70], while mTOR knockouts are embryonic lethal [72]. Thus, if PA is essential for mTOR activity, then other sources of PA must be utilized. The two candidate compensatory pathways for PA production into mTOR are LPAAT and DGK pathways. Of interest, LPAAT-derived PA is derived from exogenously provided fatty acids.

mTOR has long been known to respond to amino acids, glucose and energy. However, lipids are another essential nutrient, and sensing mechanism on sufficiency of lipid precursors is not yet known. Based on the central position of PA in lipid biosynthesis, and its involvement in mTOR regulation, we set forth to investigate the requirement of PA in regulating mTOR as a more evolutionary primitive mechanism of sensing lipid precursors.

1.7 TARGETING METABOLIC PATHWAYS IN HUMAN CANCERS

Altered metabolic programs in cancer cells are crucial for supporting increased proliferation and growth. However, metabolic reprogramming also makes cancer cells more dependable on their new rewired metabolic network. Thus, one of the emerging fields of cancer therapeutics is the possibility of targeting the special metabolic needs of cancer cells [5,
There has been considerable enthusiasm about the possibility of interfering with both glucose [105] and glutamine [29] utilization as therapeutic options for human cancers. However, strategies that target enhanced dependence on glucose in cancer cells have been so far not very successful [106, 107]. Thus, there is a clear need to explore other metabolic processes that could be therapeutically targeted.

Interfering with fatty acid metabolism has received some attention. Most of the studies target de novo synthesis of FA in cancer cells [92, 108], and FASN has emerged as an attractive target for cancer therapy, and several compounds are known to inhibit FASN. These include cerulenin, C75, orlistat, C93 and naturally occurring polyphenols. However, overexpression of FASN in many tumors has created FASN inhibition ineffective [109]. Although lipids could be synthesized in the proliferating cells, a portion of lipids are acquired from bloodstream. There has been very little reported on the utilization of exogenously supplied lipids and the therapeutic options of inhibition of lipid uptake.

The most likely way to produce cytotoxic effect is by creating synthetic lethality, or blockage of several pathways that cells depend on. mTOR integrates signals that respond to nutrients and promotes cell cycle progression and cell survival [110]. We have previously reported that suppression of mTOR in the absence of serum results in apoptosis in cancer cells harboring mutant Ras genes in the absence of serum [111-113]. Here, we identify an enhanced need for exogenously supplied serum lipids in Ras-driven human cancer cell lines that creates a synthetic lethality[114] for suppressing mTOR. This finding could be therapeutically targeted in what may be as many as 30% of all human cancers.
CHAPTER II
METHODS AND MATERIALS
2.1 CELLS, CELL CULTURE CONDITIONS

The MDA-MB-231, Calu-1, BJ, MCF7, BxPC3, T24, HT29, Panc-1, HCT116 cell lines used in this study were obtained from American Type Culture Collection. Cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma) supplemented with 10% FBS (Sigma). BxPC3 cell line was maintained in Roswell Park Memorial Institute (RPMI) (Sigma) medium supplemented with 10% FBS. Delipidated FBS was obtained from Gemini Bio Products (900-123).

2.2 MATERIALS

Reagents were obtained from the following sources. Antibodies against Cleaved PARP, actin, Akt, P-Akt (Ser473), P-Akt (Thr308), S6 kinase, P-S6 kinase (Thr389), 4EBP1, P-4EBP1 (Thr37-46), FASN, SCD1, ACL were obtained from Cell Signaling; antibodies against KRas were obtained from Abcam. MTT reagent was obtained from Sigma. Rapamycin was obtained from LC Labs, and EIPA was obtained from Sigma.

2.3 LIPID MIX SUPPLEMENTATION

Fatty acid mix was obtained from Invitrogen (11905) and was supplied to cells as 1:200 dilution complexed with 10% BSA (Sigma) in 2 to 1 ratio for the final concentration of lipids in the media of 0.375 mg/L. The exact composition of the fatty acid mixture is provided in Table S1. Palmitic acid (Sigma) was diluted in Pluronic F-68 (Gibco, 24040) and supplied to the cells in the complex with BSA to the final concentration of lipids of 0.2 mg/L. The reduced level of lipid used was due to cytotoxicity of higher concentrations palmitic acid.
2.4 TRANSIENT TRANSFECTIONS

Plasmids for transient transfections were obtained from the following sources: constitutively active KRas (Missouri S&T cDNA Resource Center, RASK2000C0), pcDNA3.1 vector (Invitrogen). For transient transfections, cells were plated at $3 \times 10^3$ cells/6-well plate (cell proliferation assay) or at 30% confluence/60-mm plate (flow cytometry assay), overnight, and transfected using Polyfect (Qiagen) according to manufacturer’s instructions. 18 hours post-transfection, cells were shifted to conditions as described in the text and figure legends.

2.5 CELL PROLIFERATION

Cells 6-well plates at 3,000 cells per well in 2 ml of full serum media overnight and shifted to various lipid conditions the next day. Media was not changed throughout the course of the experiment. After 5 days, cell number was determined by counting viable (adherent) and non-viable (floating) cells using hemocytometer. The MTT cell viability/growth assay, which measures NAD(P)H dependent anabolic activity was performed according to the vendors (Sigma) instructions.

2.6 PLD ACTIVITY

PLD was determined by accumulation of the transphosphatidylation product $[^3H]$-phosphatidylbutanol as described previously [51]. Lipid membranes were labeled with $[^3H]$-myristic acid (60 Ci/mmol; 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.

2.7 WESTERN BLOT ANALYSIS

Proteins were extracted from cultured cells using M-PER (Thermo Scientific, 78501). Equal amounts of proteins were subjected to SDS-PAGE on poly-acrylamide separating gel, and the electrophorezed proteins were transferred onto nitrocellulose membrane. The nitrocellulose membrane containing proteins was blocked in 5% non-fat dry milk solutions in PBST. Membranes were incubated in primary antibodies overnight, and secondary antibodies for an hour. Detection of proteins was performed using ECL system (Thermo Scientific).

2.8 FLOW CYTOMETRIC ANALYSIS

Cultured cell were washed and collected via trypsinization. Recovered cells were resuspended in the solution containing 7 ml of 2% bovine serum albumin in phosphate buffered saline, 5mM EDTA, 0.1% NaN$_3$ and fixed by drop wise addition of 3ml of 100% ethanol. Fixed cells were collected and resuspended in 500 µl of sorting buffer containing 2% bovine serum albumin in phosphate buffered saline, 0.1% Triton-X 100, 5mM EDTA, 40µg/ml propidium iodide, 100µg/ml RNAse A, and incubated at 37°C 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).
2.9 IMMUNOPRECIPITATION

Cells were grown in 6-well plates. Before lysing, cells were washed with cold PBS and lysed on ice for 5 min with 100µl of ice-cold 0.3% CHAPS IP buffer (40 mM HEPES [pH 7.5], 120 mM NaCl, 1mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 0.5 mM orthovandate, protease and phosphates inhibitors). Approximately 200 µg of protein was incubated with appropriate antibodies for 4 hours, and immunoprecipitated were recovered using protein G sepharose. The immunoprecipitates were later subjected to Western blot analysis along with total cell lysate.

2.10 LIPID UPTAKE VISUALIZATION

Cells were grown on 25-mm sterile, acid-etched plates containing glass coverslip until 50% confluency was reached. For the lipid uptake, cells were washed with Hank’s solution, and incubated in serum-free media with fluorescently tagged lipid vesicles (20 µM BODYPY 500/510C₁₂ (Invitrogen), 20 µM lipid mix in delipidated BSA (1:1 molar ratio) for 30 min at 10 ºC, and rinsed with Hank’s solution twice to remove unbound lipid vesicles. Warm serum-free media was added for 5 min immediately before visualization using Nikon E800 spinning disc confocal microscope using X60 objective.
2.11 PA SUPPLEMENTATION

Right before supplementation, the appropriate amount of PA was dried under N\textsubscript{2}, and resuspended by vortexing and sonicating in D-PBS for 5 minutes. The resulting PA suspension was immediately added to cell culture to the final concentration of 100µM. Due to short half-life of PA, this process was repeated every one hour throughout treatment.
CHAPTER III
Ras-DRIVEN CANCERS DISPLAY INCREASED SENSITIVITY TO LIPID WITHDRAWAL
3.1 LIPID DEPRIVATION LEADS TO INCREASED PLD ACTIVITY IN Ras-DRIVEN HUMAN CANCER LINES

We previously reported that serum withdrawal led to an increase in phospholipase D (PLD) activity that was largely restricted to cancer cells harboring Ras mutations [111]. PLD generates phosphatidic acid (PA) from phosphatidylcholine, which is required for the stability and activity of mTOR complexes [48, 51]. mTORC1 is a sensor of nutrients that regulates both cell cycle progression and survival [115]. It has been suggested that activation of the mTOR signaling node is the most commonly dysregulated signal in human cancers [10, 116]. Since PA is at the center of membrane phospholipid biosynthesis, we recently proposed that PA impacts upon mTOR as an indicator of sufficient precursors for membrane synthesis in dividing cells and that cancer cells have co-opted PLD to sustain cell proliferation and survival of cancer cells by providing the PA needed to keep mTOR active [117]. Thus, we speculated that the increased PLD activity observed in K-Ras-driven cancer cells could be a response to insufficient lipids in serum. To test this hypothesis, we examined the impact of serum lipids on the PLD activity stimulated by the withdrawal of lipids from MDA-MB-231 breast, Calu-1 lung, and T24 bladder cancer cells – all of which harbor mutant Ras genes and were previously shown to elevate their PLD activity in response to serum withdrawal [111]. Replacing 10% fetal bovine serum (FBS) with 10% delipidated FBS resulted in an increase in PLD activity in all three cell lines that was almost as robust as that observed with complete withdrawal of FBS (Figure 8). If a mixture of fatty acids (FAs) consisting of saturated, mono-unsaturated, and poly-unsaturated FAs (Table 1), was substituted for the FBS, elevated PLD activity was still observed – indicating that the lack of serum growth factors also contributed to the elevated PLD activity. Importantly, if the delipidated FBS was combined with the FA mixture, PLD activity was restored to the basal level
observed in the presence of serum. These data demonstrate that the elevated PLD activity observed in response to serum withdrawal in Ras-driven human cancer cells is due in part to the absence of serum lipids.

Figure 8. Elevated PLD activity in response to serum withdrawal in Ras-driven cancer cell lines is dependent on withdrawal of both growth factors and lipids. MDA-MB-231, T24, and Calu-1 cells were plated at 70% confluence overnight, and shifted for 24 h to various media conditions containing 10% FBS, 0% FBS, 10% delipidated FBS, 0% FBS with BSA-FA mixture, 10% delipidated FBS with BSA-FA mixture. After 20 hr, [3H]-myristic acid was added for 4 hr to label lipids. 1-BtOH was added for 20 min, and the amount of the PLD catalyzed transphosphatidylolation product, phosphatidyl-butanol, was determined as described in Experimental Procedures. Values were normalized to the levels of PLD activity in full growth serum conditions, which were given a value of 100%. Error bars represent S.D. values for at least two independent experiments.
Elevated PLD activity promotes survival in response to stress in Ras-driven cancer cells [111]. To test whether the increased PLD activity in Ras-driven cell lines yields in proliferative advantage, we investigated the dependence on serum lipids of a subset of human cancer cell lines for cell proliferation and viability. MDA-MB-231 breast and Calu-1 lung cancer cells both harbor K-Ras mutations; whereas MCF-7 breast cancer cells and the diploid human fibroblast BJ hTERT cells do not. Cells were plated and after 24 hr were shifted to the indicated conditions. After 5 days, viable and non-viable cells were counted. For all three cancer cell lines, there were very few viable cells after 5 days when put in medium lacking FBS (Figure 9). In contrast, almost all of the BJ cells were still viable. If the cells were put in medium containing 10%
delipidated FBS, virtually all of the Ras-driven MDA-MB-231 and Calu-1 cancer cells, were non-viable, whereas the MCF7 and BJ cells were mostly viable – indicating a significant difference in the way that Ras-driven cancer cells respond to the lack of serum lipids. Most strikingly, when the FA mixture was provided to the K-Ras-driven MDA-MB-231 and Calu-1 cells, there was a dramatic increase in cell viability relative to the viability observed with delipidated FBS – indicating a greater dependence on lipids than growth factors for both proliferation and survival (Figure 9). Both the MCF7 and BJ cells had lower cell numbers with the FA mixture than with the delipidated FBS – indicating a greater dependence on growth factors than lipids for proliferation. The combination of delipidated FBS and the FA mixture restored full proliferation and survival to all cell lines. Of interest, a lipid mixture that contained only palmitic acid (PA), the sixteen carbon saturated fatty acid produced by de novo fatty acid synthesis, did not enhance either survival or proliferation – indicating that the longer chain unsaturated fatty acids present in the FA mixture were critical. Similarly, bovine serum albumin (BSA), which was included as a carrier for the FA’s, did not improve survival of the MDA-MB-231 or Calu-1 cells in 10% delipidated FBS. The pattern of a greater dependence of lipids than growth factors for Ras-driven cancer cells was also observed with a larger panel of human cancer cell lines (Figure 10).
**Figure 9.** Ras-driven cancer cells are uniquely dependent on exogenous fatty acids. MDA-MB-231, Calu-1, MCF7 and BJ cells were plated and shifted to media conditions as indicated and cell number was determined 5 days later. Each measurement was normalized against the cell number for cells grown in 10% FBS, which was given a value of 100%. Attached viable cells are in blue, detached non-viable cells are in red. Error bars represent S.D. values for at least two independent experiments.
Figure 10. Ras-driven cancer cells are uniquely dependent on exogenous fatty acids. MDA-MB-231, Calu-1, HCT116, HT29, T24, Panc1, BxPC3, MCF7 and BJ cells were plated and shifted to media conditions as indicated and cell number was determined 5 days later. Each measurement was normalized against relative cell number in full growth serum condition, which was given a total value of 100%. Error bars, S.D. values for at least two independent experiments.
We next compared the effect of serum deprivation and serum deprivation + FAs on cell cycle progression using flow cytometry. All four cell lines (BJ, MCF7, MDA-MB-231, and Calu-1) had increased G1 DNA content when cells were shifted from 10% FBS to 0% FBS (Figure 11, left panel). However, if the cells were shifted to 0% FBS in the presence of the FA mixture, the BJ and MCF7 cells still arrested in G1, whereas the K-Ras-driven MDA-MB-231 and Calu-1 cells did not significantly accumulate in G1. Similarly, there was a reduction of cells in S-phase for all cell lines when placed in 0% FBS (Figure 11, right panel). However if the FA mixture was provided, there was still a reduction in S-phase cells in the BJ and MCF7 cells, but not so in the MDA-MB-231 and Calu-1 cells. Raw cell cycle profile numbers of cell types screened are depicted in Table 2. These data demonstrate that the MDA-MB-231 and Calu-1 cells harboring K-Ras mutations could sufficiently resume normal cell cycle with addition of FA alone. In contrast, the MCF7 and BJ cells accumulated in G1 in the absence of growth factors.

![Figure 11. Re-addition of FA in the absence of growth factors leads to normal cell cycle profile in Ras-driven cells.](image)

Changes in G1 and S phase cell population were evaluated by flow cytometry. MDA-MB-231, Calu-1, MCF7 and BJ cells were plated at 30% confluence in 10% FBS and shifted to 0% FBS with or without BSA-FA mix. DNA content/cell was evaluated 48 hours later using flow cytometric analysis as described in Experimental Procedures. Values were normalized to cell cycle profile in full serum condition and relative difference is plotted as indicated.
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<td>45.5±4.2</td>
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Table 2. Cell cycle profile in Ras-driven cells. Cell lines were plated at 30% confluence in 10% FBS and shifted to lipid conditions as indicated. DNA content/cell was evaluated 48 hours later using flow cytometric analysis as described in Experimental Procedures.
and the addition of lipids did not make a difference. Thus, K-Ras-driven cancer cells have a greater dependence on exogenously supplied lipids and a lesser dependence on serum growth factors than the BJ and MCF7 cells.

To further demonstrate that mutant Ras promotes a dependence on lipids, we transfected the BJ cells with a plasmid expressing oncogenic K-Ras (K-Ras$^{V12G}$). Parental BJ cells transiently transfected with K-Ras-expressing and empty vector plasmids were shifted to medium containing 0% FBS and 0% FBS containing the FA mixture and the relative cell numbers were determined three days later. The cells transfected with the K-Ras$^{V12G}$ plasmids had significantly fewer cells in the absence of FBS than the control BJ cells transfected with vector alone (Figure 12). However, if FAs were included with the medium lacking FBS, then the Ras$^{V12G}$-transfected BJ cells grew as well as the parental BJ cells – indicating that expression of K-Ras$^{V12G}$ sensitized the BJ cells to the lack of lipids in the media.

Figure 12. Expression of K-Ras$^{V12G}$ sensitized the BJ cells to the lack of lipids in the media. BJ cells were transfected with constitutively active K-Ras (V12G) or empty vector as described in Experimental Procedures, and shifted to full growth serum media or to 0% serum media with or without BSA-FA mix at 18 hours post-transfection. 72 hours later, cell number was determined. Each measurement was normalized against relative cell number in 10% FBS, which was given a value of 100%. Error bars represent S.D. values for at least two independent experiments.
3.3 ONCOGENIC Ras PREVENTS INDUCTION OF STEAROYL-CoA DESATURASE-1 UPON SERUM AND LIPID WITHDRAWAL

The above results clearly indicate a differential response to lipid deprivation in cells expressing mutant Ras. Key enzymes in the generation of fatty acids needed for membrane biosynthesis include fatty acid synthase (FASN), ATP citrate lyase (ACL), and stearoyl-CoA desaturase-1 (SCD1) [108]. We examined the effect of serum and lipid withdrawal on the levels
of these three enzymes in the BJ, MCF7, and MDA-MB-231 cells (Figure 13a). While no significant changes in expression levels of FASN and ACL were detected in response to serum or lipid withdrawal, there was a dramatic increase in the level of SCD1 in the BJ and MCF7 cells. In contrast, the MDA-MB-231 cells did not elevate SCD1 expression in response to serum and lipid deprivation (Figure 13a). Similarly, if the K-Ras$^{V12G}$ expressing plasmid was transfected into BJ cells, it suppressed the elevated expression of SCD1 observed in response to serum withdrawal (Figure 13b). Although the effect is more pronounced for serum withdrawal than lipid withdrawal, the lack of response in cells with oncogenic K-Ras indicates that these cells have a disabled response to serum and lipid deprivation.
Figure 13. Oncogenic Ras leads to ablation of stearoyl-CoA desaturase-1 levels.  
a, MDA-MB-231, Calu-1, MCF7 and BJ cells were plated and shifted to the indicated media conditions as indicated for 18 hours at which time lysates were collected and immunoblotted with the indicated antibodies. Two exposure times are shown for SCD1 in order to reveal the weak response by the MDA-MB-231 cells.  
b, BJ cells were transfected as in C, and shifted to serum free conditions for 18 hours at which time the levels of FASN, ACC, and SCD1 were evaluated by Western blot analysis. Data are representative of two independent experiments.
CHAPTER IV
LIPID WITHDRAWAL CREATES SYNTHETIC LETHALITY FOR RAPAMYCIN IN Ras-DRIVEN CANCERS
4.1 WITHDRAWAL OF SERUM LIPIDS CREATES SYNTHETIC LETHALITY FOR RAPAMYCIN IN CANCERS HARBORING MUTANT Ras.

We previously reported that in the absence of serum, MDA-MB-231, as well as most other cancer cells, are killed by rapamycin treatments capable of suppressing phosphorylation of the mTORC1 substrate eukaryotic initiation factor 4E-binding protein-1 (4E-BP1) [112, 113] (Figure 14a). In the presence of serum, cells are protected by TGF-β present in serum, which prevents apoptosis by inducing G1 cell cycle arrest [113, 118] (Figure 14b). However, while the MDA-MB-231 cells were protected from the apoptotic effect rapamycin by TGF-β alone, these cells were not completely healthy and there was significant sub-genomic DNA indicating some level of apoptosis [113]. Thus, there was apparently something else in the serum that along with TGF-β contributed to survival. We therefore examined the effect of serum lipids on the effect of rapamycin on cell viability of MDA-MB-231, BJ, and MCF7 cells. As reported previously, in the absence of serum, rapamycin (20 µM) induced cleavage of the caspase 3 substrate poly-ADP-ribose polymerase (PARP) in the MDA-MB-231 cells – indicating apoptotic death (Figure 15a). PARP cleavage was not observed in the presence of 10% FBS. PARP cleavage was not observed with rapamycin treatment in the BJ or MCF7 cells in either the presence or absence of serum. Most significantly, if cells were deprived of lipids, by incubating in 10% delipidated serum, rapamycin still induced PARP cleavage – indicating that the lack of lipids created a synthetic lethal phenotype for rapamycin treatment. If the FA mix was provided in the absence of FBS, rapamycin still induced apoptosis – consistent with our previous observation that TGF-β was required for suppressing rapamycin-induced apoptosis in the presence of serum. If the FA mixture was combined with the delipidated serum PARP cleavage was suppressed.
Figure 14. High dose rapamycin, previous findings. a, Effects of high-dose rapamycin are due to the suppression of 4E-BP1 phosphorylation and the suppression of eIF4E (adapted from [90]), b, TGFβ suppresses rapamycin-induced apoptosis in MDA-MB-231 cells (adapted from [91]).

Stimulation of apoptosis, indicated by induction of PARP cleavage in the delipidated serum required the high dose of rapamycin (Figure 15b) that causes a complete dissociation of mTOR from the mTORC1 companion protein Raptor and suppresses 4E-BP1 phosphorylation [112].
We also examined the effect of rapamycin on MDA-MB-231 cells deprived of lipids on cell viability/growth using the MTT assay. The high dose rapamycin reduced cell viability in the absence of FBS (Fig. 16a), in delipidated FBS, and in the presence of the FA mixture – but not in the presence of delipidated FBS + FA mixture. The effect of rapamycin on cell viability on MCF7 and BJ cells was substantially less than that observed for the MDA-MB-231 cells (Figure 16a). The loss of cell viability, like PARP cleavage, required the high dose rapamycin treatment (Figure 16b). Thus, the K-Ras-driven MDA-MB-231 cells are sensitized to rapamycin by depriving cells of either serum lipids or growth factors.

Figure 15. Protection of rapamycin-induced apoptosis by serum requires both lipids and growth factors. a, MDA-MB-231, MCF7 and BJ cells were plated at 70% confluence overnight and shifted to media conditions as indicated containing 20uM rapamycin. Cell lysates were collected 4 hours post-treatment, and lysates were immunoblotted with the indicated antibodies. All data are representative of at least two independent experiments, b, MDA-MB-231 cells were plated at 70% confluence overnight and shifted to 10% delipidated FBS media conditions containing rapamycin dose as indicated. Cell lysates were collected 24 hours post-treatment, and lysates were immunoblotted with the indicated antibodies.
The sensitivity of Ras-driven cancer cells deprived of serum lipids to rapamycin suggests a means to target the many cancers that harbor Ras mutations. However, ridding the serum of lipids in a human would be problematic. However, it could be possible to block the uptake of serum lipids. Dafna Bar-Sagi and colleagues demonstrated previously that mutant Ras stimulates pinocytosis [119]. We therefore examined whether blocking pinocytosis would also sensitize the Ras-driven MDA-MB-231 cells to rapamycin. Pinocytosis is suppressed by 5-(N-ethyl-N-isopropyl) amiloride (EIPA) [120]. We examined the effect of EIPA on the rapamycin
sensitivity of MDA-MB-231, Calu-1, MCF7, and BJ cells in the presence of 10% FBS. EIPA treatment made the MDA-MB-231 and Calu-1 cells sensitive to the apoptotic effect of rapamycin (Figure 17a), while having no such effect on the MCF7 or BJ cells. Another indicator of apoptotic cell death is the appearance of sub-genomic DNA. We compared the levels of sub-genomic DNA in the BJ and MDA-MB-231 cells treated with EIPA and rapamycin. The combination of EIPA and rapamycin induced a substantial increase in sub-genomic DNA in the MDA-MB-231, but not the BJ cells (Figure 17b, Table 4). Importantly, EIPA treatment alone had no significant effect on viability of cells.

We also examined whether EIPA could sensitize the BJ cells expressing activated K-Ras cells to rapamycin treatment. The BJ-KRas cells displayed PARP cleavage when treated with the combination of EIPA and 20 µM rapamycin (Figure 18). Thus, EIPA, like lipid deprivation, creates a synthetic lethal phenotype for rapamycin in K-Ras-driven cancer cells.

**Figure 17.** Blockage of micropinocytosis mimics lipid deprivation and sensitizes Ras-driven tumors to mTOR inhibitors. a, MDA-MB-231, Calu-1, MCF7 and BJ cells were plated overnight and shifted to 10% FBS conditions containing EIPA or rapamycin as indicated. Cell lysates were collected 18 hours post-treatment. Data shown is the representative blot of at least two experiments. b, MDA-MB-231 and BJ cells were plated at 40% confluence and treated as in (a) for 48 hours, after which collected cells were subjected to flow cytometric analysis. Total sub-genomic amount is plotted as indicated. Error bars, S.D. values for at least two independent experiments.
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<td>11.31±3.71</td>
<td>6.57±1.23</td>
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</table>

Table 4. Cell cycle profile of BJ and MDA-MB-231 cells showing subgenomic DNA level when exposed to mTOR inhibitor, rapamycin, and EIPA. MDA-MB-231 and BJ cells were plated at 40% confluence and shifted to 10% FBS conditions containing EIPA or rapamycin as indicated for 48 hours, after which collected cells were subjected to flow cytometric analysis.

![Figure 18. Blockage of micropinocytosis mimics lipid deprivation and sensitizes Ras-driven tumors to mTOR inhibitors.](image)

BJ cells were transfected with constitutively active KRasV12G or empty vector and treated with EIPA or rapamycin in 10% FBS condition as described in (17a). Cell lysates were collected 18 hours after treatment and subjected to immunoblot analysis with antibodies as shown. Data shown is the representative blot of at least two experiments.
4.3 POSSIBLE MECHANISM OF LIPID UPTAKE IN Ras-DRIVEN CELLS

Fatty acids, which are insoluble in water, associate with either albumin, to create so-called plasma free fatty acid (FFA), or are esterified and packaged as lipoprotein complexes together with triglycerides, cholesterol and phospholipids, and these complexed structures enable their transport in the plasma [121]. There are several pathways that allow uptake of various serum lipid species. Cell surface receptors allow uptake of whole lipoprotein complex via clathrin-dependent endocytosis [122]. Alternatively, cells could obtain free fatty acids through work of two FA translocases, scavenger receptor A (SR-A) and CD36 via saturable protein-facilitated process [123]. Additionally, lipids could be taken up through non-specific macropinocytosis that involves engulfing of extracellular material that contains lipid droplets [124, 125]. The relative contribution to the uptake by each of the three way is not known. However, when large amounts of lipids need to be taken up, lipoprotein receptor, FA translocases and pinocytosis pathways are involved in internalization of lipids [126].

It had been reported that Ras mutation induces increased ruffling and macropinocytosis in cell, and that it leads to increased fluid endocytosis [119, 127]. The functional role of macropinocytotic uptake is not fully elucidated, however recent report has shown that increased pinocytotic uptake leads to significant internalization of album to support increased need of cancer cells for glutamine as anaplerotic source [25]. Here, we hypothesized that increased rate of pinocytosis in Ras-driven cells is used as a transport mechanism for their unique metabolic need for exogenously supplied FA.

To determine whether oncogenic Ras does indeed lead to increased philopodia formation in cells in our hands, we compared the amount of philopodia is MDA-MB-231 cells to BJ cells.
Live cells were stained with lipid marker, BODYPY 500/510C\textsubscript{12}, and live images were taken using confocal microscope. In the absence of serum stimulation, MDA-MB-231 cells have a higher number of filopodia (not shown) comparative to BJ cells. In addition, the observed filopodia are highly dynamic (not shown), as described previously: cells internalized approximately 25% of their cell volume during the course of active pinocytosis [128]. To confirm that increased filopodia formation is indeed due to activated Ras, we transiently transfected BJ cells with a plasmid expressing oncogenic K-Ras (K-Ras\textsuperscript{V12G}). The cells transfected with the K-Ras\textsuperscript{V12G} plasmids had significantly higher amount of filopodia-like protrusions compared to the control BJ cells transfected with vector alone (Figure 19). We have previously used EIPA as a pinocytotic inhibitor. Our preliminary observations show that EIPA treatment leads to decreased number and to shorter filopodia.

**Figure 19. Increase in filopodia amount is due to oncogenic Ras expression.** Cells were grown on 25-mm sterile, acid-etched plates containing glass coverslip until 50% confluence was reached. 500/510C\textsubscript{12}, BJ cells were transfected with constitutively active KRa\textsubscript{sV12G} or empty vector and cells visualized by stained with the cytoplasmic marker BODYPY.
CHAPTER V
LIPID SENSING BY mTOR
5.1 NATURE OF THE FA INFLUENCES mTOR RESPONSE

It has been reported that dietary lipids stimulate S6K1 phosphorylation \textit{in vitro} and \textit{in vivo}, and that this effect is independent of amino acid stimulation of mTOR [129]. We have recently proposed that PA serves as a sensor of lipid sufficiency to mTOR. Recent reports had shown that mTOR is being differentially activated depending of the saturation level of PA species. PA species with two saturated palmitates had been shown to inhibit mTORC2 [95], while 1-palmitoyl, 2-oleoyl-PA is stimulatory for both mTORC1 and mTORC2 [51, 96]. Thus, some degree of desaturation on PA is needed for activation of mTOR. Given the link between oncogenic Ras and sensitivity to lipid withdrawal, we sought to compare responsiveness of mTOR substrates in MDA-MB-231 and BJ cells when placed in various lipid conditions. As shown in Figure 20, S6K1 phosphorylation was sensitive serum and lipid withdrawal (0% serum condition) in BJ and to lesser extend in MDA-MB-231 cells, however lipid withdrawal alone has very little effect on phosphorylation level of S6K1 in both cell types. Re-addition of mixture of FA stimulated S6K1 phosphorylation in BJ cells slightly, indicating that in normal cells, for complete mTORC1 activation, growth factor stimulation is necessary. Lipid withdrawal had a more drastic effect on the phosphorylation level of Akt at the mTORC2-dependent site S473. Lipid withdrawal led to significant reduction of phosphorylation levels of Akt (S473) in BJ cells while having almost no effect on MDA-MB-231 cells (Figure 20). FA re-stimulation led to slight increases in phosphorylation level of Akt (S473) in both BJ and MDA-MB-231 cells. Importantly, re-stimulation with palmitic acid alone had inhibitory effect on Akt phosphorylation in both cell types. This observation suggests that if PA species that feed into mTOR come from dietary lipids, then some level of unsaturation is necessary for the activation of the mTOR,
consistent with the reported finding that di-palmitoyl PA leads to inhibition of mTORC2 [95]. Taken together, these data indicate that Ras-driven cells exhibit lessen responsiveness to lipid withdrawal compared to normal cells. These data further support apparent insensitivity of Ras-driven cells to exogenous lipids due to concurrent increase in PLD activity that supplies mTOR with PA to keep metabolic machinery going when lipid resources are scarce.

![Figure 20. Nature of FA and cell type influences mTOR response.](image)

MDA-MB-231 and BJ cells were plated and shifted to the indicated media conditions as indicated for 24 hours at which time lysates were collected and immunoblotted with the indicated antibodies. Data are representative of two independent experiments.
5.2 EIPA TREATMENT LEADS TO DISSOCIATION OF MTORC1 COMPLEX, FURTHER ENHANCING EFFECT OF RAPAMYCIN

EIPA treatment in sense mimics lipid withdrawal. However, an unexpected result of EIPA treatment is reduced levels of S6K1 phosphorylation and Akt (S473) phosphorylation in MDA-MB-231 and BJ cells (Figure 21) and other cell lines (data not shown).

Figure 21. Inhibition of mTOR with EIPA treatment. MDA-MB-231 and BJ cells were plated overnight and shifted to 10% FBS conditions containing EIPA or rapamycin as indicated. Cell lysates were collected 18 hours post-treatment. Data shown is the representative blot of at least two experiments.

We have previously reported that nano-molar rapamycin concentration disrupts the association between mTOR and raptor, and the weakening in mTORC1 structure association was sufficient to abolish S6K1 phosphorylation [51, 112]. PA has been shown to stabilize mTOR complexes [51], however, the nature of PA lipid moieties influences activation or inhibitory
effect on mTOR [51, 95, 96]. Because EIPA mimics lipid withdrawal, we hypothesized that EIPA treatment could lead to increased local concentration of inhibitory di-palmitoyl species of PA, leading to dissociation of mTOR complexes. We therefore examined the association of mTOR and raptor in the presence of EIPA and EIPA/rapamycin combination. MDA-MB-231 and BJ cells where treated in full serum condition with high dose rapamycin, EIPA or combination of both, and relative association level of mTOR with its accessory proteins, raptor and rictor, was evaluated. High dose of rapamycin led to partial dissociation of both mTORC1 and mTORC2 in full serum condition compared to untreated control (Figure 22). Importantly, EIPA addition alone was sufficient to induce weakened structure of mTORC1 and mTORC2 (Figure 22). Moreover, combinatory EIPA/rapamycin treatment led cumulative decrease in the association of both mTOR complexes. Taken together, these data support the hypothesis that dietary-derived lipids influence mTOR association, and that mTOR complexes are more sensitive to rapamycin in absence of lipids is due to initial weakened interaction.
Mammalian cells could produce palmitic and stearic acid from glucose or glutamine as a carbon bone precursor. Palmitic and stearic acid could be desaturated by SCD-1 to form palmitoic and oleic acids respectively. However, we have observed expression of oncogenic Ras leads to significant decrease of SCD1 expression (Figure 13). EIPA treatment leads to blockage of macropinocytotic uptake of FA, and presumably, increasing the concentration of palmitic acid in the cells. In order to directly test if association between mTOR complexes is disrupted due to
lowered PA concentration or change in structure of PA species when treated with EIPA, we examined whether effect could be reversed by addition of PA. As shown in Figure 23, the activation of mTORC1 was rescued with concurrent addition of EIPA and palmitoyl-oleyl-PA, but not di-palmitoyl-PA.

![Figure 23. Supplementation with mono-unsaturated PA but not saturated PA leads rescue of EIPA-induced mTORC1 activity.](image)

MDA-MB-231

<table>
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<th></th>
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Data shown is the representative blot of at least two experiments.
CHAPTER VI
DISCUSSION
DISCUSSION

Over past decade, metabolic alterations in cancer cells have received a substantial amount of interest. It had been established that cancer cells undergo a significant amount of metabolic alterations, and some of these alterations are similar to those in normal highly proliferative cells [19]. However, it is becoming more apparent that many of the metabolic alterations are specific to particular oncogenic signaling pathways [22]. Although altered metabolic machinery makes cancer cells more efficient at promoting growth when nutrients are supplied at the sufficient amounts, the dependency of cancer cells on particular metabolic reprogramming deems cancer cells susceptible to disruptions within metabolic network. Thus, the identification of metabolic weaknesses of cancer cells create a platform for therapeutic interventions.

6.1 Ras-DRIVEN CELLS ARE HIGHLY DEPENDENT ON EXOGENOUS SUPPLY OF FA

The data presented here reveal an enhanced requirement for lipids in human cancer cells harboring activating Ras mutations. Depriving these cells of lipids leads to what we would call a “replicative cell death” – continued attempts to proliferate in the absence of sufficient nutrients. Whereas most cells are capable of synthesizing fatty acids for membrane phospholipids from glucose or glutamine [91, 108], the Ras-driven cancer cells apparently have a greater need for exogenously supplied lipids. This unique property of Ras-driven cancer cells is apparently an Achilles’ heel for Ras-driven cancer cells in that suppressing mTOR in these cells induces apoptosis if the uptake of lipids is suppressed.

Of significance, two very recent reports have also identified the need for exogenously supplied protein as an amino acid supply [25] and for lipids [130] in Ras-transformed cells.
uptake of albumin served as a source of glutamine for needed for cell growth [25]. We used albumin as a carrier for exogenously supplied lipids to promote cell survival and rapamycin resistance. The use of albumin alone did not promote survival by itself (Figure 9) – indicating that while Ras-transformed cells depend on scavenged proteins as an amino acid source for cell growth, it was the lipids that were critical for survival and resistance to rapamycin. The lipid requirement for exogenous lipids by Ras-transformed cells was dependent on an unsaturated fatty acid [130]. Consistent with this, the saturated fatty acid palmitic acid was not able to substitute for the mixture of fatty acids used in this study, which consisted with several unsaturated fatty acids.

6.2 ONCOGENIC Ras ALTERS EXPRESSION LEVEL OF SCD1

The dependence of Ras-transformed cells on exogenously supplied nutrients over standard de novo synthetic pathways appears to be a Ras-driven program shift. In response to the lack of serum lipids, cells lacking mutant Ras showed a dramatic increase in the level of SCD1 (Figure 13). This is consistent with the recent report from the Rabinowitz and Thompson labs where they showed that Ras-transformed cells were resistant to inhibition of SCD1. Although they did not look at SCD1 protein levels, their study also demonstrated that SCD1 was not important for the growth of Ras-transformed cells. It is likely that the lack of increased SCD1 expression in the Ras-transformed cells is a reflection of the apparent scavenger program stimulated by Ras that involves macropinocytosis [119].

Our data showed that induction of SCD1 expression was stimulated after 18 hours of lipid withdrawal, indicating that levels of SCD1 are modulated at the rate of transcription [131]. These data is consistent with previous reports, and promoter region of SCD1 contains a number
of transcription factor binding sites [132], including sterol response element-binding protein (SREBP). However, the mechanism of regulation of SCD1 expression and modulation of its levels are not fully elucidated [133]. Interestingly, one study reported that when levels of SCD1 are down-regulated, there is concurrent up-regulation of levels of fatty acid transporter CD36 [134]. In this study we show that Ras-driven cells have an increased rate of pinocytosis that leads to higher rate of lipid uptake, to compensate for need for unsaturated fatty acids when SCD1 levels are low. Thus, we show the correlation between the levels of SCD1 and rate of macropinocytotic lipid uptake.

6.3 LIPID WITHDRAWAL AND RAPAMYCIN CREATES SYNTHETIC LEthal EFFECT ON Ras-DRIVEN CELLS

One of the greatest challenges of cancer therapy is finding a strategy that would specifically target cancer cells while being minimally toxic to the rest of the organism. Metabolic reprogramming and metabolic dependencies in cancer cells provides an exceptional platform for novel therapeutic approaches. Here, we shown that cancer cells harboring Ras mutation are uniquely sensitive to rapamycin treatment when deprived of lipids.

The rapamycin sensitivity of Ras-transformed cells deprived of lipids or with suppressed macropinocytosis may be a reflection of the impact of lipid deprivation on cell cycle progression. We reported previously that in the absence of serum, rapamycin induced apoptosis in the MDA-MB-231 cells, but in the presence of serum, rapamycin induced a TGF-β-dependent G1 cell cycle arrest that protected the cells from apoptosis [113, 135]. However, if cells were synchronized in early S-phase, then the cells were killed by rapamycin – even in the presence of serum/TGF-β. These studies indicated that if cells get past the TGF-β-dependent G1 cell cycle
checkpoint and enter S-phase, then suppression of mTORC1 activates an apoptotic program. Consistent with our previous reports, the increase in sub-genomic DNA observed in Figure 17b corresponded with large drops in the percentage of cells in S-phase and G2/M cells relative to the drop in G1 cells (Table 2). While these data are too preliminary to draw any firm conclusions about why rapamycin kills K-Ras-driven cancer cells when deprived of lipids, the data are consistent with an apoptotic effect on cells that have progressed into S-phase. It is possible that depriving K-Ras-driven cancer cells, which suppresses de novo fatty acid synthesis \[130\], of serum lipids leads to the arrest or slowed progression through S-phase. Once a cell has entered S-phase, the cell has committed to replicating its genome and doubling its mass. If mTORC1 is suppressed in S-phase cells – telling the cell that there are not sufficient raw materials to finish the job, then a default apoptotic program is activated rather than try to remedy the situation at this phase of the cell cycle. While there is still much to be learned about the impact of mTORC1 suppression on cells in S-phase, it is clear that in K-Ras-driven cancer cells, depriving cells of lipids, creates a synthetic lethal phenotype for rapamycin treatment that could create therapeutic strategies for targeting the large number of human cancers that harbor Ras mutations.

6.4 DIETARY LIPIDS DISPLAY AN ACTIVATORY EFFECT ON mTOR

In addition to need for glucose and amino acids as building blocks for macromolecular synthesis during cell growth and proliferation, actively dividing cells require exogenous or de novo synthesized lipids as precursors for membrane biosynthesis. Although most cancer cells display enhanced uptake of the nutrients, glucose and glutamine among most studied, from the environment, there is also observed concurrent aberrant response to sufficiency sensing of macromolecular precursors as noted with continuous proliferation during stress of nutrient
insufficiency. Not surprisingly, PI-3K/mTOR pathway that is responsible for checking of nutrient sufficiency in cells is one of the most commonly mutated pathways in cancer [10, 11].

mTOR has been implicated as an integrator of nutrient sufficiency and stress in cells and is activated by amino acids and glucose. However, cells also require lipids for membrane biosynthesis and survival. We have recently proposed that mTOR serves as sensor of lipid sufficiency through a lipid metabolite necessary for mTOR stability and activity, PA [56]. Of interest, we have previously shown that Ras-driven cancers display an elevated PLD activity in response to serum withdrawal, and that the increase in PLD activity is necessary for cell survival [77, 111]. Here, we have shown that PLD activity is increased in Ras-driven cells when deprived of lipids alone (Figure 8). It could be suggested that the increase in PLD-generated PA might temporarily supply mTOR with PA for mTOR activation to keep metabolic machinery running in the event of stress of nutritional insufficiency.

Here, we set forth to determine the effect of lipid stimulation on downstream targets of mTOR in normal and Ras-driven cells. We have observed that oncogenic Ras gives the cells an altered responsiveness to various lipid stimuli compared to the response in most normal cells (Figure 22). Whereas in normal cell line there is a shutting down of mTORC1 and mTORC2 as observed with decreased phosphorylation levels of S6K1 and Akt (S473) when deprived of lipids alone, in Ras-driven cell line, MDA-MB-231, there are no observed changes in phosphorylation levels of S6K1 and Akt (S473) under the same condition. Taken together, these observations could be viewed as a maneuvering act of an oncogene to keep the metabolic machinery going in the event of metabolic stress. PLD activity is increased in Ras-driven cell lines in response to stress due to lipid withdrawal to feed into mTOR for temporary survival of the cells.
6.5 LIPID SENSING BY mTOR

mTOR is a central sensor of essential nutrients needed for the synthesis of macromolecules, and it has been known to respond to amino acids, glucose and energy. Another essential nutrient for cell growth and proliferation are lipids. We have recently proposed that PA feeds into mTOR as a “check” for the lipid sufficiency [56]. An amiloride, EIPA, that blocks pinocytotic lipid uptake, was used to inhibit lipid uptake. In essence, EIPA treatment mimics LPAAT blockage. LPAAT which generates PA from dietary lipids, had been shown to activate mTOR [74]. Here, we have observed that EIPA treatment blocks S6K1 phosphorylation, indicating an indirect inhibition of PA production. Addition of PA was able to rescue the effect of EIPA, further supporting PA input into mTOR for its activation. Importantly, addition of palmitoyl-oleyl-PA, but not di-palmitoyl-PA was able to rescue mTOR activation when lipid uptake was blocked by EIPA treatment.

6.6 SIGNIFICANCE

The findings reported here reveal an enhanced need for serum lipids in Ras-driven human cancer cells that creates a synthetic lethal phenotype for suppressing mTORC1. While depriving humans of serum lipids is not practical, suppressing uptake of lipids is possible and could be exploited therapeutically. It is speculated that this property displayed by Ras-driven cancer cells represents an Achilles’ heel for the large number of human cancers that are driven by activating Ras mutations.

mTOR has long been known to respond to amino acids, glucose and energy. However, lipids are another essential nutrient, and sensing mechanism on sufficiency of lipid precursors is
not yet known. Based on the central position of PA in lipid biosynthesis, and its involvement in mTOR regulation, here we show that PA feeds into mTOR as a metabolite for sensing lipid precursors. In Ras-driven cells, PLD activity increase due to lipid withdrawal may be a mechanism to keep mTOR active during metabolic insufficiency stress.

6.7 FUTURE DIRECTIONS

6.7.1 We have shown that removal of serum lipids or inhibition of lipid uptake through general macropinocytosis inhibition increased the susceptibility of Ras-driven cells to rapamycin-induced cell death \textit{in vitro}. Perhaps, the most interesting and clinically significant future direction is to confirm these finding in mice model. Amilorides are used since 1967 for treatment of cardiac ischemia and hypertentions. EIPA, and amiloride used in this study, blocks macropinocytosis and lipid uptake. Thus, suppressing uptake of lipids is possible and could be exploited therapeutically, presenting that this property displayed by Ras-driven cancer cells represents an Achilles’ heel for the large number of human cancers that are driven by activating Ras mutations.

6.7.2 We have shown that expression of oncogenic Ras correlates to inhibition of SCD1 expression. It is known that the activity of SCD1 is regulated at the level of transcription [133], however the precise mechanism of the expression is not fully elucidated. The mechanistic basis of possible Ras regulation of SCD1 expression is of much interest as well.

6.7.3 In this study we used a mixture of lipids which contained saturated and unsaturated lipids. It is of our interest to define which lipid species could rescue the lipid dependence of Ras-expressing tumor cells by supplementing media with a single lipid species (e.g., oleate, arachidonate, linoleate and/or linolenate). Additionally, an important question is to by define
which lipids are taken up via pinocytosis versus lipid transporters. We could speculate that lipid species of various levels of saturation are taken up non-specifically, however, the specificity of lipid sensing is acquired at the level of PA that feeds into mTOR.

6.7.4 PLD is not the only source of PA that could feed into mTOR. Ras-driven cells display an increase in PLD activity when stressed by growth factor and/or lipid withdrawal. Our study suggests that Ras-driven cells circumvent cell death initially when deprived of lipids by supplying mTOR with PA to keep metabolic machinery running during metabolic stress. Whether additional PA-producing enzymes, LPAAT or DGK contribute to cell survival is of interest to evaluate. These findings would further connect mTOR to control of lipid sensing through enzymes that generate PA.

6.7.5 Among other questions that are of general interest, but stand beyond the scope of this project, are:

How is Ras signaling and lipid pinocytosis connected? Does activated Ras “sense” lipid presence in the environment?

Is there a connection between PLD activity increase and inhibition of SCD1 expression?

Does SCD1 inhibition creates synthetic lethal effect in non-Ras-driven cells?

Is lipid dependency unique to cells with oncogenic Ras?
6.8 HOW IT ALL FITS WITHIN THE CELL

Perhaps, the most interesting part of the finding that there is a difference on how mTOR within the cells “senses” various sub-forms of phosphatidic acid, taken that they indeed are able to differentiate those lipid moieties.

If we start by looking at the close up view of PA binding to FBR domain of mTOR and then look at the how FRB domain fits within the total structure of mTOR complexes, it becomes clear that electrostatic interaction between lipid and protein potentially has a great effect on the mTOR complex formation as a whole.

From the crystal structure of mTOR [68], it had been revealed that phosphate group of PA binds to a positively charged patch formed by the side chain of R2109. Of a note, R2109 had been widely conserved throughout many species [56]. From the interaction of acyl chains of PA with FRB domain of mTOR, it is evident that acyl chains with unsaturated residues would bind tighter to the protein surface, considering that the surface has a bend shape at the site of PA binding. It is possible that PA with di-saturated acyl groups binds to mTOR and distorts its structure by increasing the volume of FRB domain and making it an unfavorable for mediator proteins to bind.

If we look at the mTOR crystal structure as a whole, the inhibitory effect of “swelled up” FRB domain becomes more obvious. It appears to be that FRB domain forms a cap on the top of the kinase domain [136]. FRB domain that forms a complex with either FKBP12-rapamycin or with rigid di-saturated PA species could occupy an additional space, making the cap and the gap for downstream targets two small for the interaction with kinase domain.
However, how do mTOR and PLD and PA fit together within the real estate of the cell? It had been shown that PLD 2 localizes almost exclusively at the plasma membrane, while PLD1 is found throughout the cell, but primarily in perinuclear, Golgi, and heavy membrane fractions [137]. mTOR had been shown to be localized to the plasma membrane, lysosomes, mitochondria, cytoplasm, peroxisomes, and nucleus [138]. Hence, mTOR and PLD potentially co-localize. The interaction between the phosphatidic acid and mTOR presumably occurs at the plasma membrane, which limits FRB domain accessibility to one of the acyl groups of PA[68]. However, membrane interaction could also mean that the regulation of mTOR by PA occurs via targeted localization of the active complex.

mTOR localization lies vis-à-vis to its activation, and, perhaps, phosphatidic acid is the molecular zip-code on where mTOR decides to dock and activate.
CHAPTER VII
WORKS CITED


