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LIPID SENSING BY MAMMALIAN TARGET OF RAPAMYCIN

by

DEEPAK MENON

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2017

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Lipid Sensing By Mammalian Target Of Rapamycin

By

Deepak Menon

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in
satisfaction of the dissertation requirement for the degree of Doctor of Philosophy

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ABSTRACT

Lipid Sensing by Mammalian Target of Rapamycin

By

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Mammalian target of Rapamycin (mTOR) is a protein kinase that integrates nutrient and growth factor signals to promote cellular growth and proliferation. mTOR exists in two complexes - mTORC1 and mTORC2 that are distinguished by their binding partners and signaling inputs. mTORC1 is responsive to growth factors, amino acids and glucose and is associated with Raptor; whereas, mTORC2 is responsive primarily to growth factors and is associated with Rictor. Raptor and Rictor confer substrate specificity to mTORC1 and mTORC2 respectively. Phosphatidic acid (PA), a lipid second messenger and a central metabolite for membrane phospholipid biosynthesis, is required for the stability and activation of both mTORC1 and mTORC2. The negatively charged head group of PA interacts with positively charged Lys and Arg residues in the FRB (FK-506 binding protein-12 (FKBP12)-rapamycin binding) domain of mTOR and stabilizes the mTOR complexes. The PA-binding site on mTOR is highly conserved from yeast to humans – indicating the importance of PA for mTOR function.

While much is known about the role of amino acid sensing by mTOR, much less is known how mTOR responds to other essential nutrients needed for cell growth. An under-appreciated component of serum needed for cell growth is lipids that are used for the synthesis of membranes and organelles. Because of an increased utilization of exogenous lipids by KRas-driven cancer cells, we examined the effect of exogenously supplied lipids on mTORC1 and

mTORC2 in KRas-driven cancer cells. We demonstrate that both mTORC1 and mTORC2 are responsive to dietary unsaturated fatty acids through the *de novo* synthesis of PA. Fatty acids entering the cell are activated through an ATP dependent linking of Coenzyme A (CoA) by Acyl-CoA synthetase, and thereby shunting them towards *de novo* PA synthesis. We find that a specific isoform of Acyl-CoA synthetase longchain (ACSL)-5 is overexpressed in KRas-driven cancer cells. Genetic ablation of ACSL5 inhibits oleate-mediated activation of mTORC1 and mTORC2 through reduction in levels of PA. Further, inhibition of ACSL5 leads to a G1 cell cycle arrest in Ras driven cells. The activation of mTOR by oleic acid was also dependent on lysophosphatidic acid acyltransferase that adds the CoA-charged fatty acid to 1-acylglycerol-3-phosphate to generate PA. Of significance, the glycerol-3-phosphate that gets acylated to PA was derived by reduction of the glycolytic intermediate dihydroxyacetone phosphate – indicating that glucose is also sensed by mTOR via PA. In response to lipids, we observed that mTORC2 co-localizes with the mitochondrial fraction where it activates downstream targets to regulate mitochondrial metabolism. While it has long been appreciated that mTOR is a sensor of amino acids, this study reveals that mTOR also senses the presence of lipids and glucose via production of PA.

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TABLE OF ABBREVIATIONS

ACL	ATP-Citrate Lyase
ACS	Acyl CoA Synthetase
ACSL	Acyl-CoA synthetase long
ACSS	Acyl- CoA synthetase
AMPK	Adenosine monophosphate-activated protein kinase
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CDK	Cyclin dependent kinase
CM	Complete medium
CoA	Coenzyme A
DG	Diacylglycerol
DGK	Diacylglycerol Kinase
DHAP	Dihydroxyacetone phosphate
ER	Endoplasmic reticulum
FATP	Fatty acid transport proteins
FRB	FKBP-12 rapamycin binding domain
G3P	Glycerol -3- phosphate
GAP	GTPase – activating protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factors
GLUT	Glucose transporter type
GPAT	Glycerol-3-phosphate acyl transferase
GPD	Glycerol-3-phosphate dehydrogenase
GRP	Guanyl nucleotide releasing protein
GTP	Guanosine triphosphate
HK	Hexokinase
LPAAT	Lysophosphatidic acid acyl transferase
LPA	Lysophosphatidic acid
MAM	Mitochondrial Associated Membranes

mTOR	Mammalian target of rapamycin
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
PA	Phosphatidic acid
PBS	Phosphate Buffered Saline
PC	Phosphatidyl choline
PDK	Phosphoinositide dependent kinase
PE	Phosphatidyl ethanolamine
PEP	Phosphoenolpyruvate
PG	Phosphatidyl glycerol
PH	Pleckstrin homology
PI	Phosphatidyl inositol
PS	Phosphatidyl serine
PKC	Protein kinase C
PLD	Phospholipase D
POPA	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate
SCD	Stearoyl CoA desaturase
SFK	Src family kinase
SUV	Small unilamellar vesicles
TCA	Tricarboxylic acid
TGF	Transforming growth factor
TPI	Triose Phosphate isomerase

Chapter 1 INTRODUCTION

1.1 The Structure of mTOR

Mammalian target of Rapamycin (mTOR) is a Ser/Thr protein kinase that belongs to the phosphatidylinositol 3-kinase-related kinases (PIKKs) superfamily that regulates mammalian cellular growth and differentiation (Ma and Blenis, 2009). mTOR is a 289 kDa protein made up of 2549 amino acids. The structure of mTOR can be divided into five major domains- HEAT (Huntingtin, elongation factor 3 (EF3), a subunit of PP2A, and TOR) repeats, FAT (FRAP, ATM, and TRRAP) domain, FRB (FKBP12 Rapamycin binding) domain, catalytic domain, LBE and FAT-C domain (Figure 1.1). The FAT, kinase and FAT-C domains are conserved amongst members of the PIKK superfamily. The N-terminus is made up of ~20 tandem HEAT repeats. Each repeat is made of ~ 40 amino acids that forms a super helical structure and helps in protein-protein interaction (Andrade and Bork, 1995). The FAT domain consists of four alpha helices that wraps around the kinase and the FRB domains (Figure 1.1). The FRB domain of mTOR is known to bind Rapamycin-FKBP12 complex that inhibits the ability of substrates to access the active kinase site and thus, affects mTOR activity (Choi et al., 1996). The FRB domain of mTOR acts as a gatekeeper preventing the entry of the substrates into the active site and Rapamycin FKBP12 acts by reducing the accessibility of the active site (Figure 1.1, B) (Yang et al., 2013). The FATC domain of TOR is required for its kinase activity and is packed against the activation loop (Figure 1.1, B). The deletion of FATC domain has been shown to completely inhibit kinase activity of TOR (Takahashi et al., 2000).

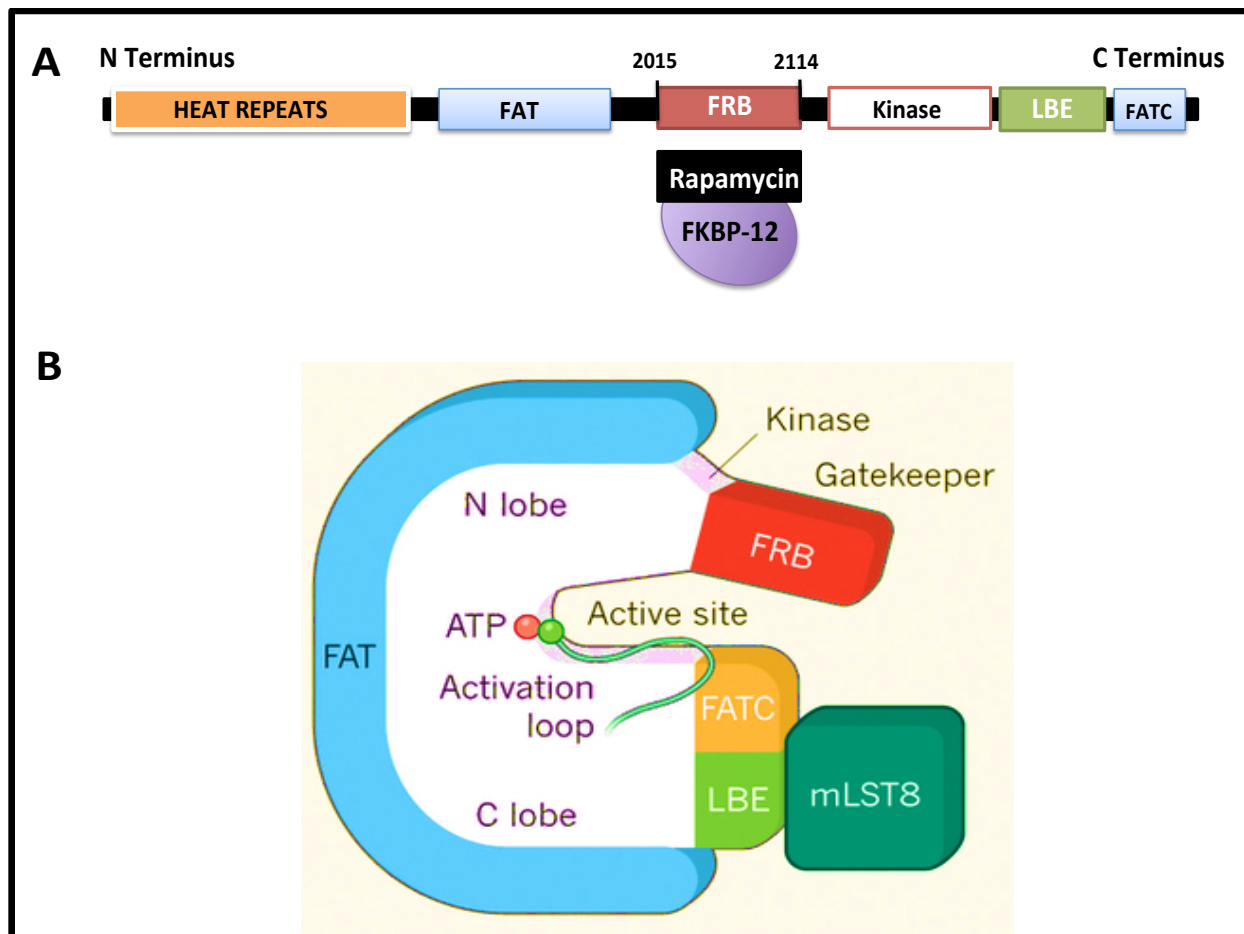


Figure 1.1: A) mTOR Domain structure B) Schematic of structural features of mTOR - adapted from (Alessi and Kulathu, 2013)

A) N terminal of mTOR consists of a set of 40 HEAT repeat domains, followed by FAT, FRB, kinase, LBE and FATC domains. Rapamycin in complex with FKBP-12 binds FRB domain of mTOR and inhibits its activity. B) Schematic showing how the domains come together to form the 3D structure of mTOR. The FAT domain wraps around the kinase domain. The FRB domain acts as a gatekeeper to prevent entry of substrates into the catalytic site of mTOR. The activation loop undergoes a conformational change upon kinase activation and is typical of PIKK family proteins.

1.2 mTOR Complexes: mTORC1 and mTORC2

mTOR derives its name from the ability of a drug Rapamycin to inhibit its activity. Rapamycin is a macrolide drug that was derived from fungal strain *Streptomyces hygroscopicus* found in the island of Rapa Nui in 1972 (Sehgal et al., 1975). Owing to its inherent immunosuppressive property it was sent to NCI for screening as a tumor suppressor. Its ability to inhibit tumor progression *in vivo* brought the drug to limelight and was declared to be of high importance. It was not until 1991 that its ability to inhibit TOR was discovered in yeast (Heitman et al., 1991). mTOR integrates external nutrient cues to cellular growth and proliferation (Laplante and Sabatini, 2012). mTOR forms two distinct complexes – mTORC1 and mTORC2 (Figure 1.2). The two mTOR complexes have differential sensitivities to rapamycin – with mTORC1 more sensitive than mTORC2 (Sabatini, 2006).

mTORC1

Amino acids, glucose and growth factors inputs activate mTORC1 complex. mTORC1 complex includes PRAS40, DEPTOR and mLST8. PRAS40 and DEPTOR are negative regulators of mTORC1 activity that competes with substrates like 4EBP-1 for binding to Raptor (Wang et al., 2007) and FRB domain respectively. mLST8 has been shown to affect the complex stability by interacting with LBE and FATC domains (Figure 1.2).

Downstream Targets of mTORC1: mTORC1 activity phosphorylates two critical downstream targets that control protein synthesis in cells – ribosomal subunit S6kinase (S6K) and eukaryotic initiation factor 4E (eIF4E) binding protein 1(4EBP-1) (Foster and Fingar, 2010) (Figure 1.2). Phosphorylation of S6K occurs at Threonine 389 by mTORC1.

In absence of mitogenic stimuli, a pool of S6K and hypo phosphorylated 4EBP-1 exists in association with eukaryotic initiation factor (eIF)-3 complex and eIF4E respectively at the mRNA 5'-cap. In response to growth factors, amino acids and glucose, mTORC1 phosphorylates S6K and 4EBP-1. This leads to dissociation of eIF3 from S6K that allows it to phosphorylate initiation factors eIF4B leading to increased translational efficiency. Hyper phosphorylated 4EBP-1 releases itself from eIF4E allowing it to form the translational initiation machinery (Hay and Sonenberg, 2004).

mTORC2

mTORC2 has been shown to be responsive to growth factor inputs (Figure 1.2). Upon activation, it forms a complex with Rictor (rapamycin insensitive component of TOR). mSin1 is an essential component of the mTORC2 complex required for its assembly and ability to activate Akt (Frias et al., 2006). DEPTOR is a negative regulator of the mTOR complex, expression of which is dependent on mTOR activity (Peterson et al., 2009). DEPTOR associates with the FRB domain and is displaced by Phosphatidic Acid (PA) allowing for activation of mTOR (Yoon et al., 2015).

Downstream Targets of mTORC2: Akt is phosphorylated at two sites for complete activation. First, phosphorylation at T308 is carried out by phosphoinositide-dependent kinase (PDK)-1 at the plasma membrane. Second, phosphorylation of Akt at Ser 473 by mTORC2 leads to complete activation that regulates processes like cellular growth, glucose metabolism and apoptosis (Manning and Cantley, 2007). Akt promotes survival by blocking pro-apoptotic proteins like BAD (Datta et al., 1997). Akt exerts its control over cellular proliferation through phosphorylation of a cyclin dependent kinase inhibitor - p27^{Kip-1} (Liang et al., 2002). Phosphorylation of p27^{Kip-1} prevents it from entering the nucleus, thereby preventing its ability to

block cell cycle progression. Serum and glucocorticoid inducible kinase (SGK1) and Protein Kinase C (PKC) are other well-known mTORC2 targets.

Besides, the role of Akt in cellular survival and proliferation, it has profound effect on metabolism. Akt has been shown to promote glucose transporter type (GLUT)-4 translocation to the plasma membrane, allowing glucose uptake in response to insulin stimulation (Kohn et al., 1996). Further, Akt stimulates glycolysis by promoting association of hexokinase (HK) with the mitochondria allowing rapid conversion of glucose to glucose-6-phosphate (G6P) (Pastorino and Hoek, 2008). Akt has also been shown to regulate *de novo* fatty acid synthesis through activation of ATP-Citrate Lyase (ACL) (Berwick et al., 2002). Akt phosphorylates ACL leading to its activation and synthesis of Acetyl CoA- precursor of both cholesterol and palmitic acid.

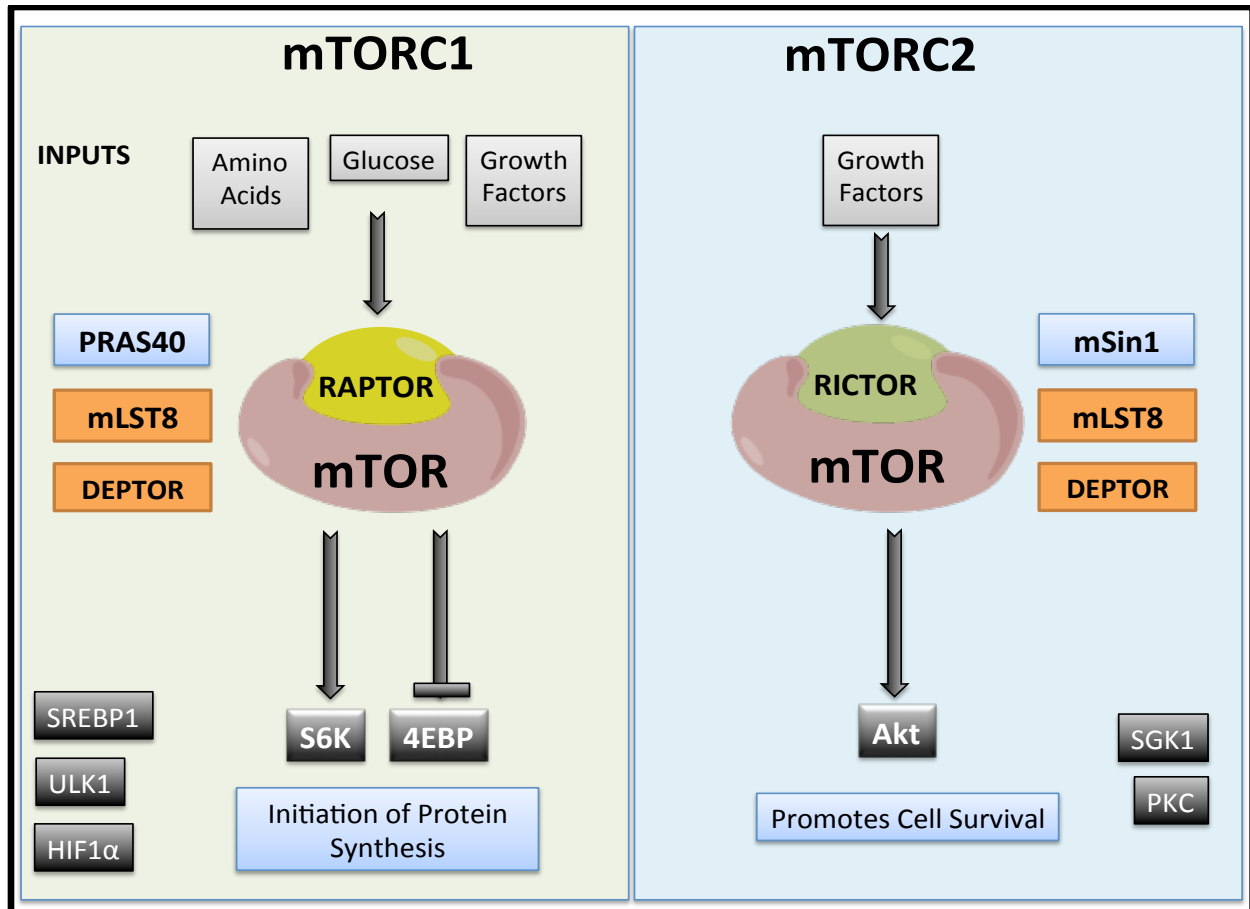


Figure 1.2: mTOR forms two distinct complexes in response to nutrient inputs.

mTORC1 and mTORC2 complexes respond to different nutrient inputs. mTORC1 is responsive to amino acids, glucose and growth factors. Upon activation by nutrients mTORC1 leads to phosphorylation and activation of its downstream targets S6K, 4EBP-1, SREBP1, ULK1 and HIF1- α . S6K and 4EBP-1 control initiation of protein synthesis. mTORC2 activation in response to growth factors leads to phosphorylation of Akt, SGK1 and PKC (Protein Kinase C). Akt activates its downstream targets that promote cell survival. Mammalian sin1 (mSin1) is a required for mTORC2 activity.

1.3 Localization of mTOR Complexes

Upon mitogenic stimuli mTORC1 and mTORC2 localize to different organelles where they encounter the substrates to be phosphorylated. Localization of these complexes is significant in terms of nutrient sensing ability and access to downstream targets. For instance, lysosomes provide a source of amino acids from degradation of internalized proteins where mTORC1 is localized (Sancak et al., 2008). In contrast, association of HK2 with the mitochondrion provides easy access to mTORC2, which has been reported to be associated with the mitochondria (Betz and Hall, 2013; van Vliet et al., 2014).

Amino acids, growth factors and glucose have been shown to regulate localization of mTORC1 complex to the lysosomal membrane. The translocation of mTORC1 upon mitogenic stimuli involves active guanosine triphosphate (GTP)-bound Rag proteins (Bar-Peled et al., 2012; Sancak et al., 2008). At the lysosomes the active GTP-bound Rheb activates mTORC1. Rheb exists at the lysosomes in an inactive GDP bound state due to the GTPase-activating protein (GAP) activity of tuberous sclerosis (TSC)-1/TSC2. Upon mitogenic stimuli Akt phosphorylates TSC1/TSC2 leading to inhibition of its GAP activity, thereby stimulating Rheb by allowing it to be in active GTP-bound state (Huang and Manning, 2008). This leads to activation of mTORC1.

Upon stimulation by growth factors mTORC2 complex has been shown to localize predominantly to the endoplasmic reticulum (ER) where it is shown to phosphorylate Akt at Ser473 (Boulbes et al., 2011). Several studies have shown Akt to be associated with the mitochondria. The downstream target of mTORC2, SGK1, is localized at the mitochondria (Engelsberg et al., 2006). More recently, Betz et al. confirmed the presence of Akt and mTORC2

complex at mitochondrial associated membranes (MAM). MAMs are regions of close associations between ER and mitochondrial membrane. MAMs are signaling hubs for calcium transport and platform of phospholipid synthesis and transfer. Most lipid biosynthetic enzymes are known to be localized at the MAMs (Voelker, 2005). It was shown that mTORC2 translocates to the MAMs upon stimulation with insulin and plays a role in maintaining mitochondrial function (Betz et al., 2013).

1.4 mTOR and the Cell Cycle

The mammalian cell cycle is divided into 4 phases – G1, S, G2 and M (Figure 1.3). G1 and G2 phases are characterized by growth and protein synthesis. S phase after the G1 phase is characterized by DNA synthesis. Cell division occurs during the M phase. The transition of cells from one phase to the next is tightly regulated by cellular signaling machinery and regulated by growth factors and nutrient availability. The signaling machinery that regulates passage of cells through the cell cycle includes cyclins and mTOR (Cuyas et al., 2014). This section discusses how cells integrate the nutrient sensing to cyclin dependent cell cycle regulation.

Cyclin D and Cyclin E regulate progression of cells through G1 phase of the cell cycle. Cyclin D in complex with cyclin dependent kinase (CDK)-4 or CDK6 can phosphorylate Retinoblastoma protein (Rb), which exists in complex with elongation factor E2F (Figure 1.3). Upon hyper phosphorylation of Rb, E2F is released allowing cells to progress to the S phase of cell cycle (Foster et al., 2010). Thus, phosphorylation status of Rb determines the fate of cell cycle progression (Figure 1.3)

As discussed earlier (section 1.2 mTOR allows for initiation of protein synthesis through activation of two major downstream targets – S6K and 4EBP-1. This allows for progression of cells from G1 to S - phase of cell cycle. Inhibition of mTOR by rapamycin arrests cells in G1 phase of cell cycle. This arrest is mediated by up regulation of transforming growth factor (TGF)- β mediated signaling and down regulation of Rb-phosphorylation through suppression of 4EBP-1 (Chatterjee et al., 2015). In all mTOR activity mediates cell growth through phosphorylation of 4EBP-1 and S6K.

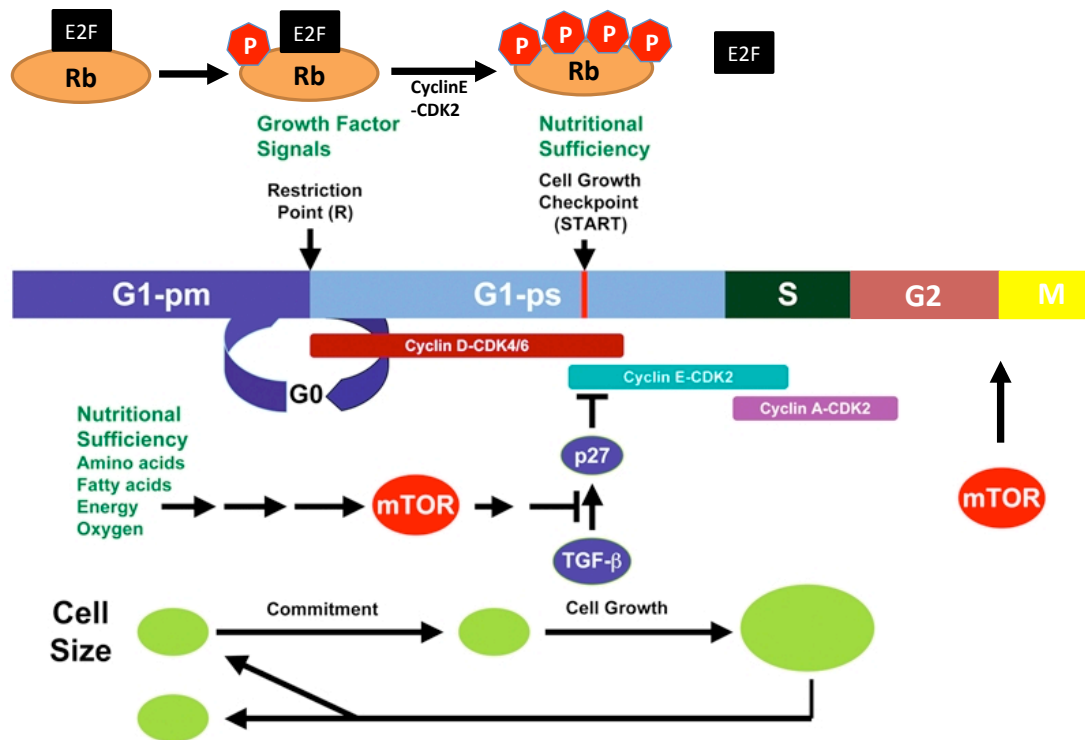


Figure 1.3: Cell cycle regulation by Cyclins and mTOR - adapted from (Foster et al., 2010)

Progression from G1 to S phase requires cells to overcome the cell growth checkpoint mediated by mTOR. mTOR by inhibiting TGF- β mediated growth arrest through activation of p27 allows cells to progress through the checkpoint into the S phase of cell cycle. Further, cyclin D-CDK4 allow progression to S phase by phosphorylating protein Rb releasing E2F. This allows for initiation of protein translation.

1.5 Phosphatidic Acid: Parent Lipid to Membrane Phospholipids

PA is an anionic lipid with a polar phosphomonoester head group and two hydrophobic fatty acid tails attached to a diacylglycerol (DG) backbone. Besides playing a structural role as a parent to most lipid moieties in the membrane (Figure 1.4), PA has multiple signaling functions. As a precursor to membrane lipid synthesis, addition of a cytidine diphosphate (CDP) head group to PA leads to formation of CDP- DG that can be used for synthesis of phosphatidylinositol (PI), phosphatidylglycerol (PG) and diphosphatidylglycerol (Cardiolipin). PA can also be acted upon by PA phosphatases to yield diacylglycerol (DG). DG can either be used for synthesis of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) via the Kennedy pathway or can be stored as triacylglycerols (Shindou and Shimizu, 2009). In mammalian cells, phosphatidylserine (PS) is synthesized by exchange of headgroups from PE. In summary, PA acts as a parent lipid to all membrane phospholipids (Figure 1.4).

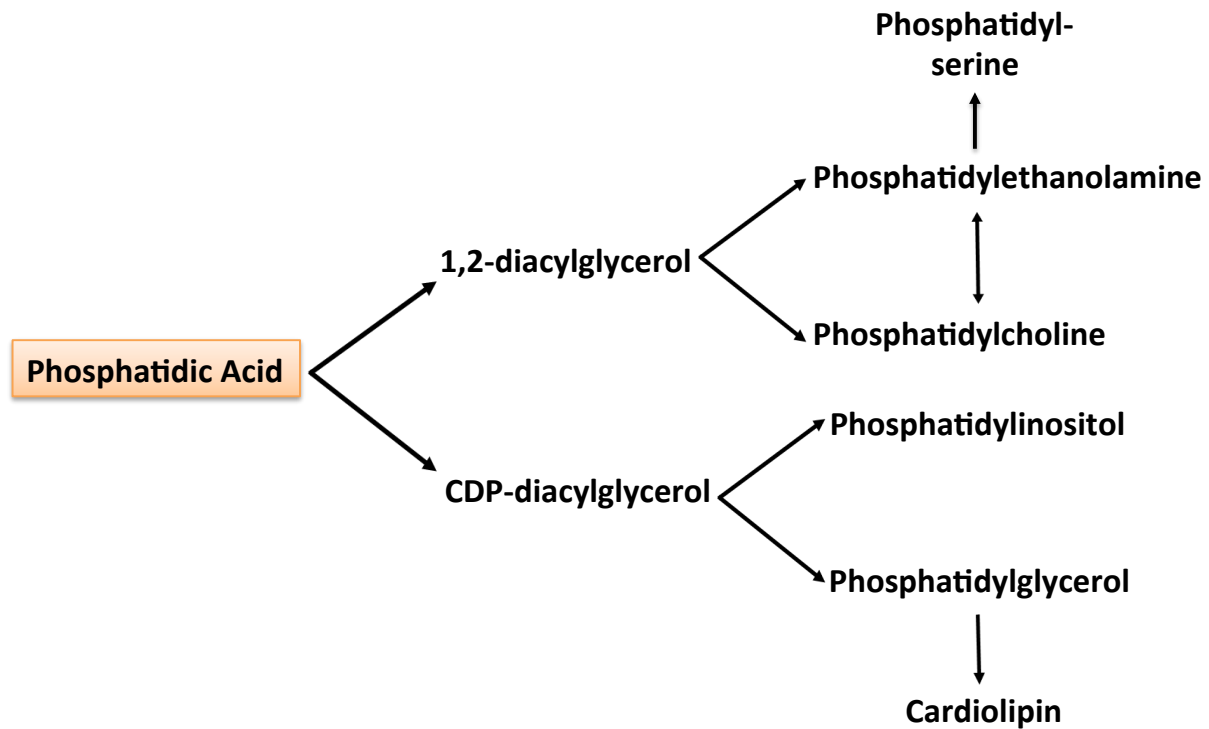


Figure 1.4: Phosphatidic acid is the parent lipid to cell membrane phospholipids.

PA can be dephosphorylated to generate 1,2 – diacylglycerol that acts as a precursor for generation of phosphatidylethanolamine, phosphatidylcholine and triacylglycerol. CDP head group addition leads to generation of CDP-diacylglycerol that is the precursor to phosphatidylinositol, phosphatidylglycerol and cardiolipin.

1.6 Maintaining PA Levels - Alternative Sources of PA Synthesis

Along with its structural function, the role of PA as a second messenger molecule underscores its importance in mammalian cell signaling pathways. Not surprisingly, various pathways regulate the generation of this important messenger molecule. Three major pathways of PA generation exist in a mammalian cell. Diacylglycerol kinase (DGK) adds a phosphate group to diacylglycerol (DG) to generate PA. Phospholipase D (PLD) generates PA through hydrolysis of PC (Figure 1.5). The *de novo* synthesis of PA occurs through the addition of two fatty acyl chains to glycerol-3-phosphate (G3P) by glycerol 3-phosphate acyl transferase (GPAT) and lysophosphatidic acid acyl transferase (LPAAT).

DG the substrate for DGK, is a second messenger known to bind target proteins like protein kinase C (PKC), and ras-guanyl nucleotide releasing protein (GRP) (Sakane et al., 2007). Thus, conversion of DG to PA is significant as it regulates DG levels in a cell. PA phosphatase can help maintain the equilibrium between DG and PA, by catalyzing the dephosphorylation of PA to generate DG. This can be critical in maintaining lipid homoeostasis (Carman and Han, 2009). Currently known DGK isozymes have been divided into 5 groups (Type I-V) based on their structures. Type I family (α , β , γ) has a EF hand motif known to bind Ca^{2+} ; Type II (δ , η , κ) has a pleckstrin homology (PH) domain; Type III lacks any recognizable domains; Type IV (ζ , ι) has a MARCKS (myristoylated alanine rich C kinase substrate) domain, Type V is characterized by the presence of a ras associating (RA) domain (Coleman and Lee, 2004; Sakane et al., 2007). Amongst the known roles of DGK generated PA, DGK α upon activation by phosphoinositide-3 kinase (PI3K) through Src family kinase (SFK), promotes G1 – S transition in T lymphocytes and therefore is a positive regulator of cell proliferation (Flores et al., 1999). This DGK isoform is highly expressed in several melanoma cell lines and overexpression of this isoform suppressed

tumor necrosis factor (TNF)- α induced apoptosis, thereby acting as a negative regulator of cell death (Yanagisawa et al., 2007). DGK inhibitor R59949 has been shown to be a catalytic inhibitor of DGK α (Jiang et al., 2000). There have been mixed reports on DGK mediated activation of mTOR and therefore warrants further study. Flores et al. have reported DGK ζ to be a positive regulator of mTOR signaling in serum deprived HEK293 cells (Avila-Flores et al., 2005). This suggests that the type I DGK isozyme DGK α plays a role in mTOR activation. .

LPAAT is involved in the last step of *de novo* PA synthesis in cells. LPAAT adds on a fatty acyl group to Lysophosphatidic acid (LPA) contributed by two different pathways (Figure 1.5). Two major LPAAT isoforms LPAAT- α and LPAAT- β have been attributed for the bulk of LPAAT activity in cells. Its activity in mammals is localized to the ER and the mitochondrial membrane (Athenstaedt and Daum, 1999). LPAAT- β plays a role in regulating adipogenesis through mTORC2 target Akt (Subauste et al., 2012). Overexpression of LPAAT- β has been reported in lung, ovarian and prostate cancers (Bonham et al., 2003; Coon et al., 2003). Recently, LPAAT- θ has been shown to activate mTOR signaling pathway (Tang et al., 2006).

Of the three pathways for PA generation, PLD generated PA has an important role in mitogenic signaling. Two mammalian isoforms of PLD are known to exist – PLD1 and PLD2 which catalyze a transphosphatidylation reaction using water to generate PA from PC (Jenkins and Frohman, 2005). PLD generated PA has been implicated in the activation of mTOR (Chen et al., 2003). PA competes with FK506 binding protein 12 (FKBP12) bound Rapamycin for binding to mTOR complexes (Chen et al., 2003; Fang et al., 2001; Toschi et al., 2009). Our lab has shown that cancer cells that harbor Ras mutations have elevated PLD activity that further rises upon serum deprivation (Shi et al., 2007; Zheng et al., 2006). Activation of mTOR through PLD generated PA is shown to protect these cells from stress induced cell death upon serum

withdrawal (Toschi et al., 2009; Zhong et al., 2003). In other words PLD generated PA acts as a survival signal. Kamphorst et al. have shown that Ras mutant cell lines are scavengers of exogenous fatty acids (Jurriaans Kamphorst, 2012). Data from our lab has shown that elevated PLD activity is actually a response to withdrawal of fatty acids present in serum (Salloum et al., 2014). In addition, mTOR activation in response to stress induced PLD activity *in vitro* (Zheng et al., 2006). It was also observed that mTORC1 activity in response to amino acid and glucose stimulation is abrogated upon PLD inhibition - indicating that PLD activity mediates nutrient input to mTOR (Xu et al., 2011). Surprisingly, PLD null mice are viable and fertile (Burkhardt et al., 2014) whereas mTOR knockout is embryonic lethal (Gangloff et al., 2004). It is therefore hypothesized that there exist other sources of PA generation that are involved in mitogenic signaling through mTOR.

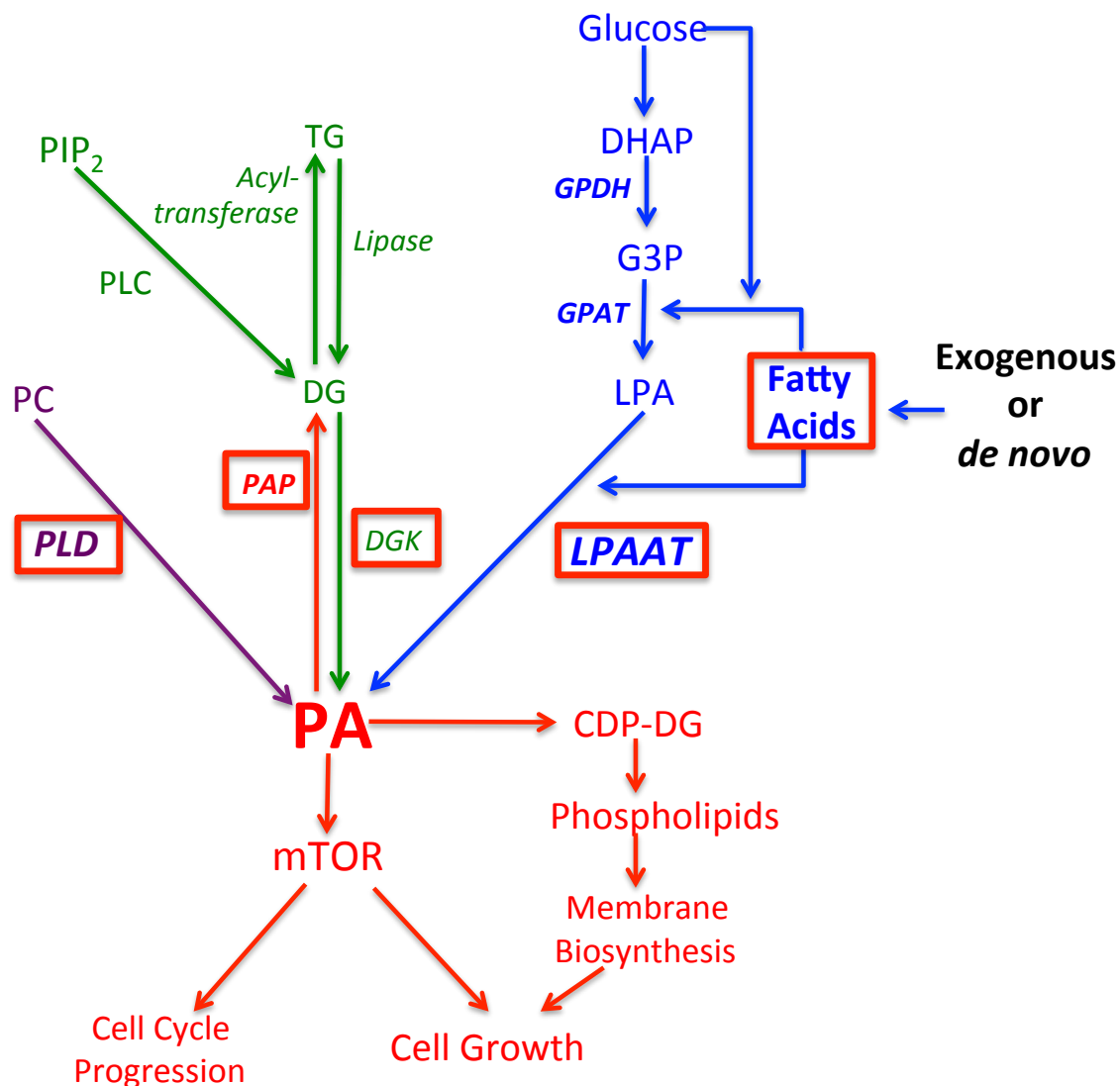


Figure 1.5: Sources and destination of PA - adapted from (Foster, 2013)

Three different enzymes synthesize PA. LPAAT adds a fatty acid group obtained exogenously or from *de novo* fatty acid synthesis to generate PA. PLD hydrolyzes PC to generate PA. DGK adds a phosphate group to DG to generate PA. PA phosphatase removes a phosphate group to convert PA back to DG, thereby maintaining PA levels in a cell. G3P derived from glycolytic intermediate DHAP provides the backbone to synthesize PA.

1.7 Phosphatidic Acid Modulates Protein Function: A pH Sensor

Besides acting as the parent lipid to all membrane phospholipids, PA is known to regulate protein function. The unique head group of PA has a pK_a between 6.9 and 7.9, and thus has a negative charge at physiological pH. This negatively charged PA head group allows it to interact with positively charged amino acids in PA binding domain of proteins via ionic interactions (Figure 1.6). Higher pH reduces the binding of protons to PA and leads to increased negative charge on the head group, enhancing the binding of PA to positively charged amino acids. This mechanism is referred to as electrostatic/hydrogen bond switch mechanism (Shin and Loewen, 2011). The negative charge on PA provides the specificity for protein binding over other phospholipids in the membrane.

One of the well-characterized PA binding proteins in yeast is Opi1, a transcription factor and a regulator of lipid metabolism in the ER. Opi1 binds PA in a pH dependent manner. At a physiological pH, the negatively charged head group of PA binds to basic residues in Opi1-Q2 domain to allow its localization at the PA rich ER membrane. Lowering of intracellular glucose perturbs the V-ATPase, inhibiting its ability to pump protons from the cytosol to the vacuolar compartments (Martinez-Munoz and Kane, 2008). A drop in intracellular pH protonates the PA head group to release Opi1 from the ER membrane and enter the nucleus to transcriptionally repress phospholipid metabolism. (Young et al., 2010).

In a study by Simons et al., siRNA mediated knockdown of sodium hydrogen exchanger (NHE)-2 transporter blocked binding of Disheveled (Dvi) protein to Frizzled membrane protein (Simons et al., 2009). Disheveled (Dvi), is a key mediator of Wnt signaling pathway that controls cellular differentiation and cell polarity in *Drosophila*. Dvi interacts with plasma membrane

through binding of basic residues in helix3 of the protein with the head group of PA in a pH dependent manner. The NHE2 sodium hydrogen exchanger creates a basic environment around the plasma membrane that generates a more negatively charged PA headgroup in the membrane and promotes the binding of Dvi to the membrane. Once at the membrane Dvi interacts with the frizzled receptor to activate Wnt signaling pathway.

Further, the composition of PA enriched membranes that interacts with the protein in question plays a major role in the binding process. The presence of PE adjacent to PA in self-assembled membranes has been shown to enhance PA binding (Shin and Loewen, 2011). Hydrogen bond between the amine of the PE with the head group of PA increases the net negative charge on the PA head group. This allows for increasing the binding affinity of PA to target proteins. The conical shape taken up by PE (Sprong et al., 2001) in membranes adjacent to PA may aid in insertion of PA enriched membrane domains into hydrophobic pockets of effector proteins.

1.8 PA Binding to mTOR

The FRB domain of mTOR has a PA binding domain that is conserved across species (Figure 1.6). Rapamycin in complex with a protein FKBP12 inhibits mTOR upon binding to FRB domain. Purified FRB domain has been shown to bind small unilamellar vesicles (SUV's) containing PA that was disrupted by addition of FKBP12-Rapamycin complex. Further, rapamycin resistant mutant of FRB domain displayed PA binding which was not inhibited upon addition of Rapamycin (Fang et.al, 2000).

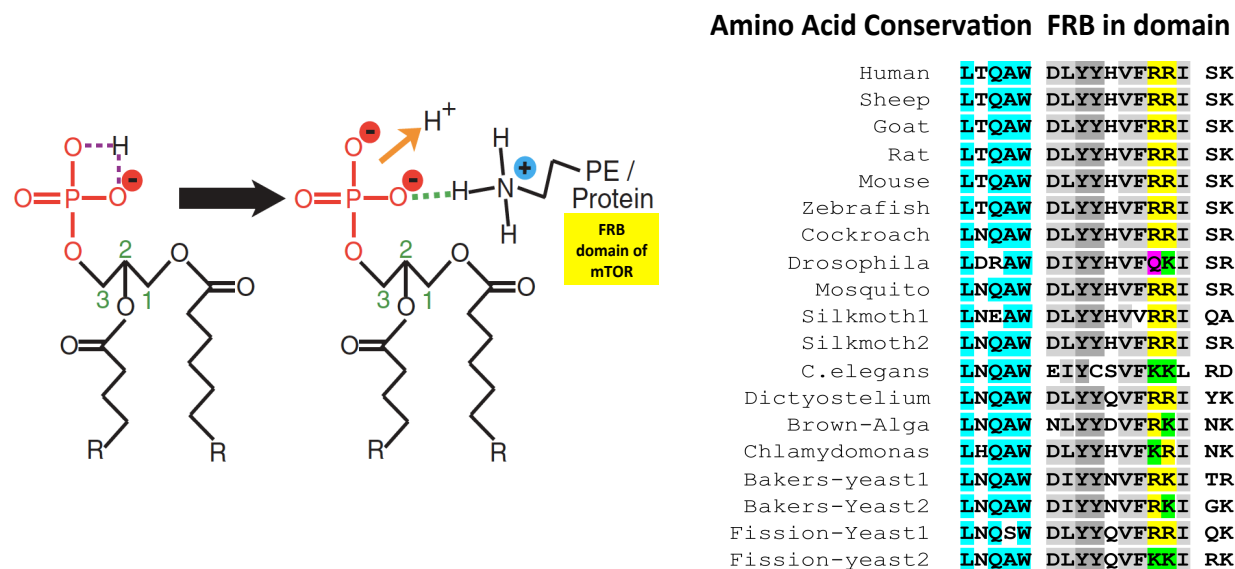


Figure 1.6: PA interacts with conserved basic amino acid residues in the FRB domain - adapted from (Foster, 2013; Shin and Loewen, 2011)

PA interacts with effector proteins through electrostatic switch mechanism via hydrogen bonding with adjacent PE residues or positively charged amino acid residues in protein. The figure on right shows conservation of amino acid residues in PA binding domain in mTOR across species.

The above observations were further strengthened by NMR structural analysis of FRB domain. The region of FRB domain that interacts with PA is lined with highly hydrophobic amino acids residues; thereby allowing it to form a channel whereby PA can enter (Figure 1.7a). There exists a hydrophobic patch formed by side chain residues (L2031, F2039, W2101, Y2105 and R2109) which binds the FKBP12-Rapamycin complex (Figure 1.7b) (Veverka et al., 2008). The NMR structure of PA bound FRB domain indicates a substantial overlap in the PA binding site and the Rapamycin binding site. The side chain of amino acid R2109 was shown to interact the head group of PA. Mutation of the key Arg residue (R2109) site to Ala on the FRB domain of mTOR has been shown to perturb PA binding to the FRB domain (Fang et. al, 2001). Of significance is the conservation of this residue across species (Figure 1.6).

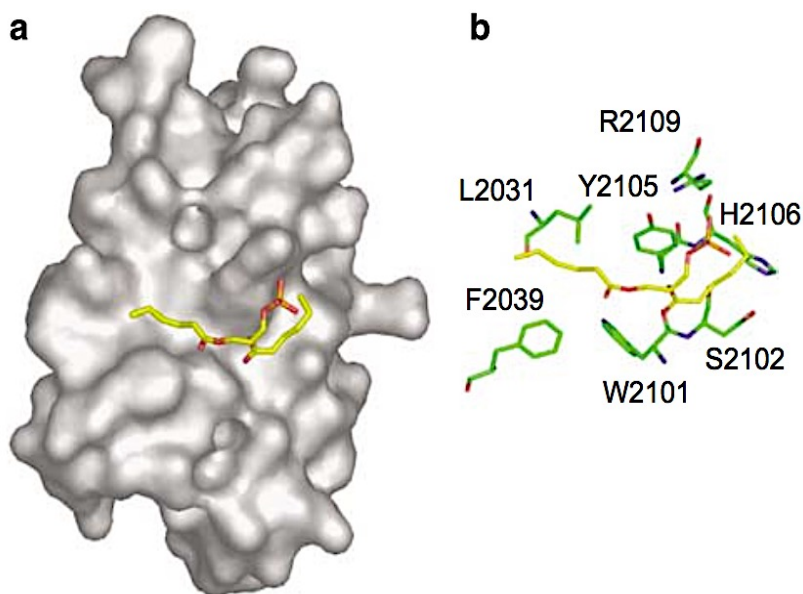


Figure 1.7: Phosphatidic acid binding to mTOR - adapted from (Veverka et al., 2008)

a) Solution state NMR structure of PA (yellow) docked into FRB domain b) Structure showing interaction of key residues of PA with an isolated FRB domain.

1.9 Metabolic Transformation Drives Cancer Cell Proliferation

Extracellular Nutrient Acquisition: Means of Cancer Cell Proliferation

Metabolic reprogramming is an emerging hallmark in cancer cell proliferation (Pavlova and Thompson, 2016). Cancer cells exhibit programmed changes in signaling pathways to maintain glycolysis, Tricarboxylic acid (TCA) cycle, phospholipid biosynthesis and redox balance. Glycolytic and lipid uptake deregulation in cancer cells are discussed below.

Glycolytic Reprogramming in Cancer: *De Novo* PA as A Glucose Sensor

Glycolytic up regulation is a means to generate ATP more efficiently than oxidative phosphorylation to sustain high rates of proliferation in cancer cells (Dong et al., 2016). Over the past decade, use of highly sensitive mass spectrometry techniques to focus on tracing path of glycolytic intermediates has allowed for discovery of novel shunts arising from the glycolytic intermediates. It is now evident that besides ATP synthesis, increased dependence on glucose can be attributed to shunting glycolytic intermediates towards anabolic processes (Figure 1.8). GLUT1 and GLUT3 transporters are overexpressed in malignancies and associated with tumor progression and decreased patient survivals (Krzeslak et al., 2012).

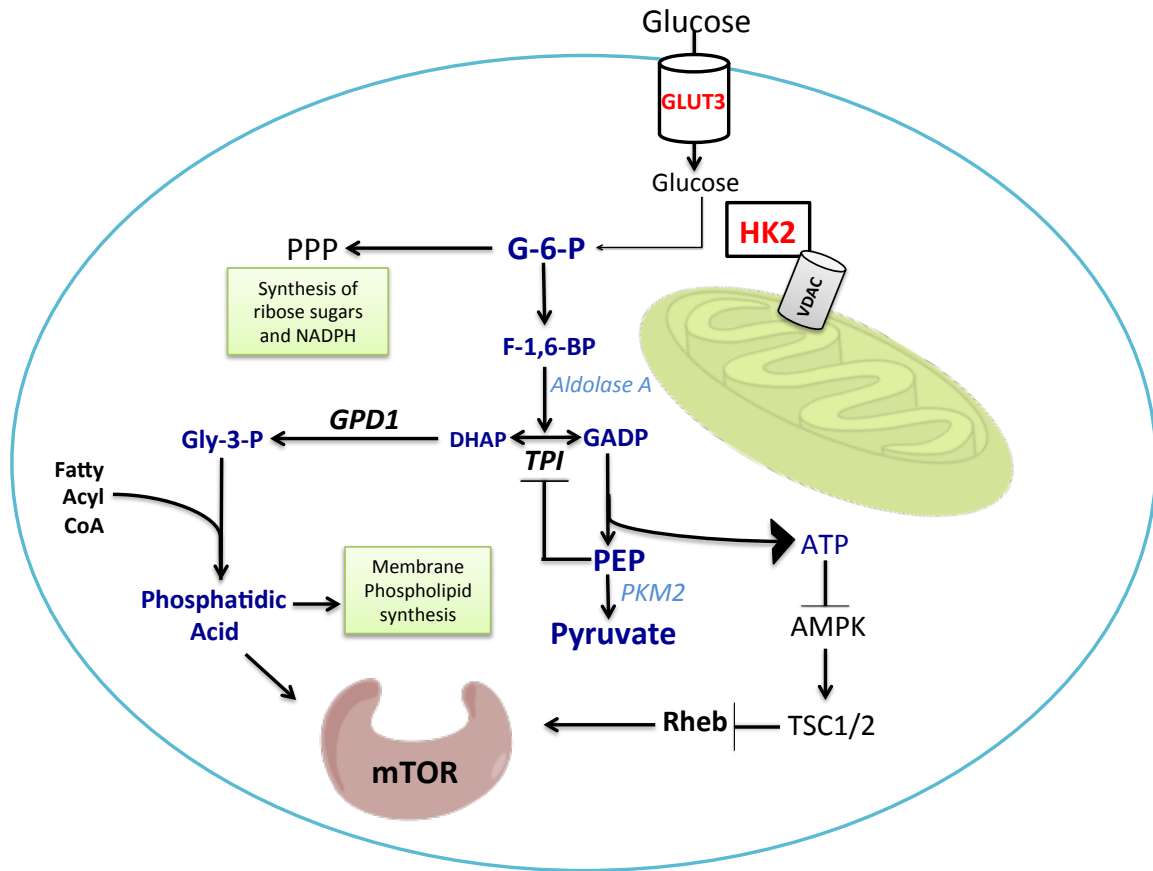


Figure 1.8: Glycolytic intermediates are reprogrammed towards anabolic pathways for cellular proliferation in cancers.

Enzymes shown red are regulated by oncogenic KRas to up regulate glycolysis. Enzymes shown in blue are dysregulated in cancers allowing flux to shift towards anabolic processes. HK2: Hexokinase 2; G-6-P: Glucose-6-phosphate; F-1,6-BP: Fructose-1,6-bisphosphate; DHAP: Dihydroxyacetone phosphate; GADP- Glyceraldehyde -3-phosphate; G3P-Glycerol -3-phosphate; PEP: Phosphoenolpyruvate; PKM2: Pyruvate kinase M2; TPI: Triose Phosphate Isomerase. G-6-P that is synthesized in the cell can be shunted towards ribose synthesis or for sustenance of glycolysis to generate adenosine triphosphate (ATP) to keep mTOR active through inhibition of adenosine monophosphate-activated protein kinase (AMPK). Glycolytic intermediate DHAP is reduced to G3P to generate PA for membrane phospholipid biosynthesis and to keep mTOR active.

Once glucose enters the cell, phosphorylation by Hexokinase generates G-6-P, a rate-limiting step that traps glucose preventing its exit from the cell. Of the known isoforms of hexokinase: HK1, HK2, HK3 and HK4 – HK2 overexpression is associated with cancers (Mathupala et al., 2006). Further, Patra et al. show that oncogenic Ras activity increases expression of HK2 thereby shifting the equilibrium towards G-6-P and allowing entry of glucose through GLUT transporters (Patra et al., 2013). G-6-P enters glycolysis to generate Pyruvate, which is transported across to mitochondria feeding into the TCA cycle. Besides the canonical pathway of ATP generation G-6-P can be shunted towards the Pentose Phosphate pathway for ribose sugar synthesis and generation of reducing NADPH for anabolic processes. F1,6 – BP in the glycolytic pathway is split into DHAP and GADP. Hepatocellular carcinomas reprogram themselves to express the highly active form of Aldolase A, thereby shifting the equilibrium to sustain glycolysis in forward direction (Figure 1.8). DHAP is a key intermediate in glycolysis that is reduced to G3P to serve as a backbone for *de novo* PA synthesis.

The glycolytic intermediate phosphoenol pyruvate (PEP) inhibits triose phosphate isomerase (TPI), which is an enzyme responsible for inter-conversion of DHAP and GADP (Gruning et al., 2011) (Figure 1.8). Cancer cells are known to express the less active pyruvate kinase isoform-PKM2 that can possibly lead to accumulation of PEP, shown to inhibit TPI (Harris et al., 2012). Accumulation of PEP leads to inhibition of TPI, which can in turn increase the levels of DHAP that can be used for PA synthesis in proliferating tumors.

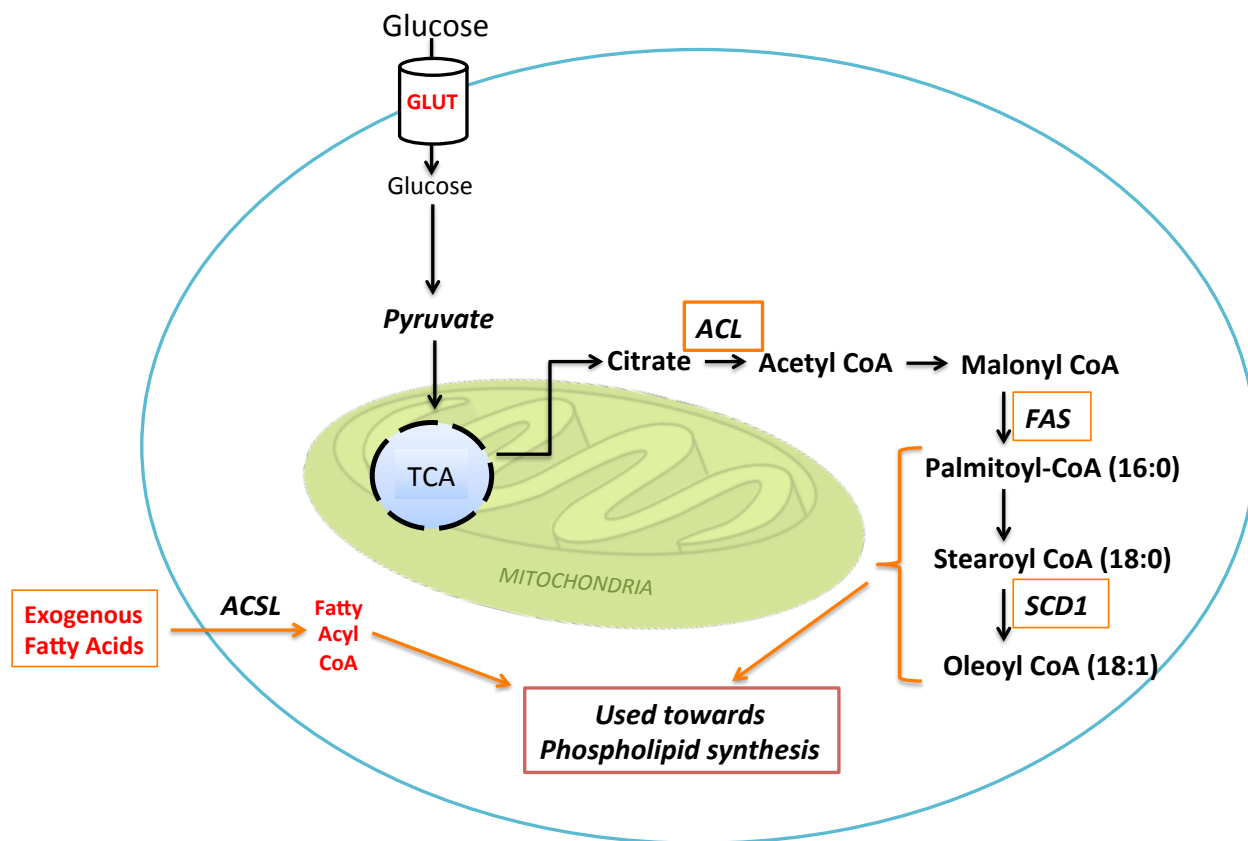


Figure 1.9: Sources of fatty acids in cells: *de novo* and exogenously supplied fatty acids

Glycolysis feeds into the TCA cycle for sustenance of mitochondrial respiration to generate ATP. TCA cycle intermediate citrate is expelled out of the mitochondria and acts as a primer for fatty acid synthesis. Citrate is converted into Acetyl CoA, which is used for synthesis of Palmitic acid (saturated) by the action of fatty acid synthase in the cell. Palmitoyl-CoA can be extended to stearate that upon being desaturated by Stearoyl-CoA desaturase (SCD)-1 generates Oleoyl-CoA. These fatty acids can then be incorporated into phospholipids. The other major source of fatty acids in culture is serum. Acyl-CoA Synthetase (ACS) can activate exogenously supplied fatty acids through addition of Coenzyme A (CoA) group. Activated fatty acids can be used for phospholipid synthesis.

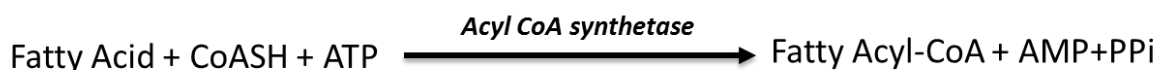
Deregulation of Fatty Acid Synthesis and Import in Cancers

The end product of glycolysis is pyruvate that enters the TCA cycle, which is the major source of ATP for non-proliferating cells. In proliferating cells, much of the citrate generated from pyruvate-derived acetyl CoA is expelled out of the mitochondria for regeneration of acetyl CoA for cholesterol and fatty acid synthesis. ATP citrate lyase (ACL) catalyzes acetyl CoA generation from citrate. Acetyl CoA generated at this step is the primer for *de novo* fatty acid and cholesterol synthesis in cells (Figure 1.9). Cancer cells are known to up regulate ACL activity in order to meet the anabolic demands for cellular proliferation (Zaidi et al., 2012). In addition, ACL activity acts a source of acetyl CoA for acetylation of several proteins that regulate cellular proliferation. ACL is a downstream target of mTORC2 substrate - Akt which stabilizes it through phosphorylation at S454 (Berwick et al., 2002). Thus, mTOR plays a significant role in regulating *de novo* fatty acid synthesis in cells through its regulation of ACL.

Cells can synthesize saturated palmitic acid, which can be extended and desaturated to generate oleic acid. SCD1 catalyzes desaturation of stearoyl-CoA to generate oleoyl-CoA (Figure 1.9). KRas-driven cells have been shown to down-regulate expression of SCD-1 and hence depend on exogenous lipids for survival (Kamphorst et al., 2013). Once fatty acids enter into the cells they are activated by addition of CoA group by ACS. This prepares the fatty acids to be incorporated into phospholipid synthesis pathways. The nature, activity and deregulation of this class of enzymes are discussed in the next section.

1.10 Acyl-CoA Synthetases: Deciding the Fate of Exogenous Lipids

In addition to *de novo* fatty acid synthesis, cancer cells acquire lipids from serum in culture or bloodstream *in vivo*. As discussed in the previous section, KRas-driven cancer cells are dependent on exogenous lipids (Kamphorst et al., 2013; Salloum et al., 2014). Depriving KRas-driven cancer cells of serum lipids in culture arrests the cells in G1 phase of cell cycle. Cells commit to programmed cell death upon long term starvation (Salloum et al., 2014). Fatty acids upon entering the cells need to be activated by addition of a CoA group (Figure 1.9). Acyl-CoA synthetases catalyze this reaction in an ATP dependent manner. Acyl-CoA synthetases convert a highly hydrophobic fatty acid entering the cell into an amphipathic moiety. This process serves a twofold purpose. Addition of CoA group prevents their exit from the cell, referred to as metabolic trapping (Fullekrug et al., 2012; Zhan et al., 2012). Second, upon addition of the CoA group these fatty acids are partitioned as a newly synthesized amphipathic molecule towards phospholipid synthesis, fatty acid oxidation, transcriptional regulation or cholesterol biosynthesis. These enzymes are located on the ER and outer mitochondrial membrane.



Acyl-CoA synthetases are classified based on their substrate specificity. Based on the length of fatty acid chains they are classified into very-long chain acyl synthetase (ACSVL), acyl-CoA synthetase long chain (ACSL), medium chain acyl-CoA synthetase (ACSM) and short-chain acyl-CoA synthetase (ACSS). More recently, it has been proposed that Acyl-CoA synthetases are involved in what is referred to as vectorial acylation (Black and DiRusso, 2007). This involves a close association of fatty acid transport proteins (FATP) and ACSL that act in synchrony to allow fatty acids to enter the cell. The thio-esterification of the fatty acids entering

the cell leads to a shift in flux allowing more unesterified fatty acids to enter the cell. It has been shown that overexpression of ACSL1 leads to an increased uptake of fatty acids from the media and is a consequence of metabolic trapping (Zhan et al., 2012). ACSL1 isoform has been shown to affect partitioning of long chain fatty acids into Triglyceride (TG) synthesis and fatty acid oxidation pathways. Specifically, it affected incorporation of stearate (18:0-CoA) into phospholipids (Li et al., 2013). In the same study Liver specific knockdown of ACSL-1 has been shown to inhibit incorporation of ^{14}C -oleate into TG (Li et al., 2009). Stable knockdown of ACSL-1 in 3T3-L1 cells, led to a decrease in re-esterification of fatty acids (Lobo et al., 2009).

Knockdown studies on rat hepatocytes have shown that ACSL3, is required for *de novo* fatty acid synthesis through reducing transcriptional activity of genes involved in hepatic lipogenesis (Bu et al., 2009). ACSL5 plays a major anabolic role in incorporation of fatty acids into biosynthetic pathways. Its expression is higher in cells with enhanced TG synthesis. siRNA mediated ACSL5 knockdown in primary hepatocytes has been shown to affect oleic acid and acetic acid incorporation into glycerolipids and lipid droplets, phospholipids and cholesterol esters (Bu and Mashek, 2010). ACSL5 has been shown to localize to both mitochondria and ER. Overexpression of ACSL5 in rat hepatoma cells has been shown to increase oleic acid incorporation into TG without affecting the fatty acid synthesis pathways (Mashek et al., 2006).

Oncogenic KRas-driven cells are known to be dependent on exogenous lipids for survival have been shown to express ACSL5 at higher levels. Further, data from Chun et al. shows that siRNA mediated knockdown of KRas in HCT-116 leads to a decrease in levels of ACSL5 (Chun et al., 2010). Another isoform ACSL3 has been recently shown to be overexpressed in KRas-driven cancers, inhibition of which reduced cellular proliferation thereby highlighting the dependence of these cancers on exogenously supplied lipids (Padanad et al., 2016).

1.11 Linking Glycolysis and *De Novo* Phosphatidic Acid Synthesis - GPD1

Glycerol-3-phosphate dehydrogenase (GPD1) is a cytoplasmic enzyme that catalyzes the reduction of DHAP to G3P (Figure 1.8). The reaction is reversible and NADH acts as an electron donor in the reaction. The protein GPD1 is cytosolic and is a part of a larger G3P shuttle between cytosol and the mitochondrion. This shuttle acts as a means to regenerate NAD⁺ in mammals. Once in the inner mitochondrial membrane, G3P can be oxidized to DHAP by GPD2, associated with a reduction of flavin adenine dinucleotide (FAD). Together, this shuttle facilitates the transfer of reducing equivalents from cytosol to mitochondria (Mracek et al., 2013).

The activity of the GPD1 acts also as a link between glycolysis and PA synthesis or TG synthesis. *De novo* PA is generated upon two consecutive acylation steps of G3P. Mutations in the gene encoding GPD1 have been linked with transient infantile hypertriglyceridemia (Basel-Vanagaite et al., 2012). This condition occurs whereby there occurs an increase TG synthesis due to a possible limitation of G3P conversion to DHAP. In such a scenario G3P can be shunted towards TG synthesis. This hypothesis is further strengthened by the study that shows that there occurs an enhanced GPD activity in adipose tissue of obese patients that allows for accumulation of TG (Swierczynski et al., 2003).

It is postulated that high rates of cancer cell proliferation demand for increased phospholipid synthesis. In such a scenario, overexpression of GPD1 in cancers allows for an increased flux towards PA synthesis (Figure 1.8). Of significance are two reports of GPD1 overexpression in cancers. GPD-1 was found to be overexpressed in protein tissue extracts from primary tumors of patients with colorectal adenocarcinomas upon comparison with normal tissue

samples (Krasnov et al., 2009). Another study showed an increased activity of GPD1 in human bladder cancer (Turyn et al., 2003).

1.12 Summary and Project Rationale

We discussed how cancer cells reprogram the metabolic machinery to meet the demands for high rates of cellular proliferation. Nutrient sensing ability of the cells is regulated through mTOR that allows cells to progress through the cell cycle checkpoints. Nutrient sensing by mTOR is a well - studied subject. While a lot is known about sensing of amino acids and growth factor input into mTOR, much less is known with regard to the sensing of lipid and glucose by mTOR. Cells can synthesize *de novo* lipids or obtain lipids from serum. We sought to study how exogenous lipids drive mTOR function in regulating cellular growth and proliferation. We hypothesize that lipid sensing by mTOR occurs through generation of *de novo* PA in cells. KRas-driven cells lack the ability to desaturate the lipids, depend highly on exogenous lipids for survival. Therefore, Ras driven cells become excellent model systems to study the impact of exogenously supplied lipids on mTOR function. The significance of this study lays in the fact that understanding the dependence on lipids in cells provides a novel therapeutic opportunity to interfere with mTOR survival signals.

Chapter 2 MATERIALS AND METHODS

2.1 Materials

Oleic acid (Sigma O3008), Linoleic Acid (Sigma L9530), Palmitic Acid (Sigma P0500), Arachidonic acid (Sigma Aldrich 10931), Fatty acid free BSA (Sigma Aldrich A7030), 18:1-16:0 Phosphatidic Acid (Avanti polar lipids 840857C), Egg Phosphatidic Acid (Avanti polar lipids 84010C), Lipid Mix- Catalog 11905031, Thermo Fisher Scientific. Antibodies for P-S6K^{Thr389} (9234), P-Akt^{Ser473} (9271), S6K (9202), Akt (9272), P-Rb (9307), T-Rb(9309), Cyclin A2(4656), Raptor (2280) and Rictor (9476) were obtained from Cell signaling; ACSL5 (HPA007162), GPD1 (HPA044620) from Atlas Antibodies; LPAAT- β (TA 323423) from Origene; and GPD2 (17219-1-AP), Actin (60008-1-Ig) from Proteintech Group.

2.2 Cells And Culture Conditions

MDA-MB-231 breast, Calu-1 lung, HepG2 liver, MCF7 breast, PC3 prostate, and 786-O renal cancer cell lines and BJ-hTERT cells were obtained from American Type Culture Collection (ATCC). Calu-1 cells were cultured in McCoy's 5A medium (Sigma M8403) containing 10% fetal bovine serum and 2mM L-glutamine (Sigma G7513). All other cells were cultured in Dulbecco's Modified Eagle's Media (Sigma D6429) containing 10% fetal bovine serum (Sigma F4135). NSCLC cell lines used were from the Hamon Cancer Center Collection (University of Texas–Southwestern Medical Center) and were maintained in RPMI-1640 (Life Technologies) supplemented with 10% fetal calf serum.

2.3 Transient siRNA Transfections

Transient siRNA transfections were carried out using Lipofectamine RNAiMAX (Thermo Fisher 13778150) as per manufacturer guidelines in Optimem (Thermo Fisher 31985070). Cells were collected for analysis at time points described in the figures. The following siRNA were used in the study. Raptor siRNA (Santa Cruz sc-44069), Rictor siRNA (Santa Cruz sc-61478), LPAAT- β siRNA (Dharmacon M003811), ACSL5 siRNA (Ambion s28549), GPD1 siRNA (Ambion s223769), GPD2 siRNA (Ambion s5979), Non-Targeting siRNA (Ambion 4390843), Non-Targeting siRNA pool (Dharmacon D-001206-13).

2.4 Quantitative RT-PCR

qRT-PCR was performed using One-Step cells Direct qRT-PCR kit (11753-100 Thermo Fisher Scientific) as per manufacturers instructions. TaqMan primers-FAM (6-carboxyfluorescein) / MGB probe specific to GPD1 (Applied Biosystems ID: Hs01100039_m1) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (4352934E) were obtained as 20X mix from Applied Biosystems. One-step qRT-PCR was performed using assays specific to GPD1 and GAPDH using 3 μ L of cell lysate in 30 μ L of reaction volume. The expression levels of GPD1 mRNA were measured relative to average of GAPDH. Thermal cycling conditions were as follows: 50°C for 15 minutes, 95°C for 2 minutes, 40 cycles of 95°C for 15 seconds, 60°C for 45 seconds. The samples were run on Step One Plus RT-PCR system from Thermo Fisher Scientific and analyzed using Step one software (Ver 2.2.2).

2.5 Measurement of Phosphatidic Acid Levels

Cells were plated in complete media and serum starved during the last 16 hr of siRNA treatment. 0.1 μM [^3H]-oleic acid (5 mCi/ml) was added to the culture for 3 hr under conditions of siRNA mediated knockdown of GPD1, GPD2, LPAAT- β and ACSL5. Cells were collected in ice-cold methanol:6N HCl (50:2) and added to the first extraction buffer (155 ml of 1M NaCl and 500 ml of chloroform). The lower organic layer was transferred to second extraction buffer (350 μl of water, 115 μl of 1M NaCl and 115 μl methanol). The radioactivity of the lower organic layer was quantified by scintillation counting, and equal radioactivity counts of total lipids from each sample was dried under nitrogen. The samples were resuspended in spotting solution (chloroform: methanol 9:1, 2 μl egg PA (10mg/ml) and run on a thin layer chromatography plate in 100 ml of the upper phase of ethyl acetate:iso-octane:glacial acetic acid:water (88:40:20:80). The plates were sprayed with En3hance spray (Perkin Elmer 6NE970C) and exposed to a pre-flashed X-ray film (Amersham 28906836) and developed after 7 days at 80°C. Bands corresponding to the PA standard were quantified using Image Studio Lite 4.0.21.

2.6 Preparation of Phosphatidic Acid Vesicles

Immediately before addition, PA (16:0-18:1) in chloroform was dried under nitrogen and re-suspended in Dulbecco's Phosphate Buffered Saline (Thermo Scientific 14190). The suspension was sonicated for 3 minutes. The resulting PA suspension was added to cells at a final concentration of 300 μ M.

2.7 Western Blot Analysis

Proteins were extracted from cultured cells in Mammalian Protein Extraction Reagent (M-PER) (Thermo Scientific 78501). Equal amounts of proteins were subjected to SDS-polyacrylamide gel electrophoresis. Electrophoresed proteins were transferred to nitrocellulose membrane. After transfer, membranes were blocked in an isotonic solution containing 5% non-fat dry milk in phosphate buffered saline containing 0.1% Tween-20. 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 horse radish peroxidase conjugated Immunoglobulin G (IgG) was used for detection using Electro chemiluminescence (ECL) system (Thermo Scientific 34080)

2.8 Flow Cytometry

Cells were harvested from plate and washed twice Phosphate Buffered Saline-Ethylene Diamine Tetra Acetic acid (PBS-EDTA) solution. Pelleted cells were resuspended in fixing solution – 7ml Phosphate Buffered Saline (PBS), 2% bovine serum albumin (BSA), 5mM EDTA. 3ml of

100% ethanol was added dropwise. Fixed cells were centrifuged and the pellet was resuspended in 500 μ L of sorting buffer – 0.1% triton X, 5mM EDTA, 40ug/ml propidium iodide, 100ug/ml RNase A, in PBS. The cells were filtered through a 70 μ m mesh to prevent cell aggregates and incubated for 45 minutes at 37 C in dark. The DNA content was analyzed by flow cytometry (Becton Dickinson fluorescence activated cell sorter (FACS) Calibur). The results were analyzed for cell percentage in each phase of cell cycle using WinCycle (Phoenix Flow Systems).

2.9 Thymidine Incorporation Assay

Cells were labeled with 1 μ Ci/ml of ³H-Thymidine for 24 hours. Cells were washed with 1ml PBS, then precipitated with 1ml 10% TCA. The precipitates were solubilized in 0.5ml of 0.5% SDS/0.5M NaOH solution. The extent of labelling was determined by using 75 μ L of the lysate in 3ml of scintillation fluid.

2.10 Mitochondrial Fraction Isolation

Isolation of the mitochondrial and cytoplasmic fractions was performed using a protocol published previously (Wieckowski et al., 2009).

Chapter 3 RESULTS

Lipid Sensing by mTOR Occurs Through De novo Phosphatidic Acid Synthesis

Cancer cells sense external nutrients as cues to divide and proliferate. Mammalian target of Rapamycin (mTOR) is a critical sensor that links external nutrient signals to cellular growth. mTOR exists in two complexes mTORC1 and mTORC2. mTORC1 is sensitive to amino acids, glucose and growth factor inputs. In contrast, mTORC2 is sensitive to growth factor inputs. Both mTORC1 and mTORC2 are sensitive to Phosphatidic acid generated by Phospholipase D (Toschi et al., 2009). While PLD is a key source of PA, two other sources of PA include Lysophosphatidic acid acyl transferase (LPAAT) and Diacylglycerol Kinase (DGK). LPAAT mediates *de novo* PA synthesis by addition of a fatty acyl-CoA to Lysophosphatidic acid (LPA). LPAAT- β isoform that preferentially adds oleyl-CoA (18:1) is overexpressed and critical for survival of ovarian cancers (Hollenback et al., 2006). mTOR activation by LPAAT- β generated PA supports proliferation of pancreatic cancer cell lines (Blaskovich et al., 2013).

Utilization of exogenously supplied fatty acids begins with addition of CoA by ACSs; a critical step that directs free fatty acids to *de novo* phospholipid generation. Several ACSs have been previously shown to be overexpressed in cancers (Monaco et al., 2010; Padanad et al., 2016; Sung et al., 2003). Amongst the known isoforms of this enzyme, ACSL1 and ACSL-5 have been shown to have substrate specificity for unsaturated fatty acids (Mashek et al., 2007). ACSL5 promotes survival of glioma cells under acidic conditions (Mashima et al., 2009). Mutant K-Ras cancers depend highly on exogenous unsaturated lipids for survival, thereby making these pathways targets of cancer therapy (Kamphorst et al., 2013; Salloum et al., 2014).

In this study, we show that exogenously supplied unsaturated fatty acids activate mTOR through *de novo* PA production. KRas-driven cancer cells that depend highly on exogenously supplied unsaturated lipids for survival, overexpress enzyme ACSL5 required for fatty acid activation,

shunting it towards PA synthesis. *De novo* PA is synthesized by addition of activated fatty acids to G3P that activates mTOR allowing cellular growth and proliferation.

3.1 mTOR Responds to Exogenous Dietary Lipids

Fetal bovine serum is a complex mixture of nutrients consisting of growth factors, amino acids, glucose and the sole source of exogenous lipids for cultured cells. Ras driven cancers are scavengers of serum unsaturated lipids that are needed for their proliferation. mTOR is responsive to nutrients including amino acids and glucose and provides a link to cell growth (Fingar and Blenis, 2004; Laplante and Sabatini, 2012). We therefore, looked at impact of exogenous lipids on mTOR, key nutritional sensor known to sense glucose and amino acid inputs thereby regulating growth.

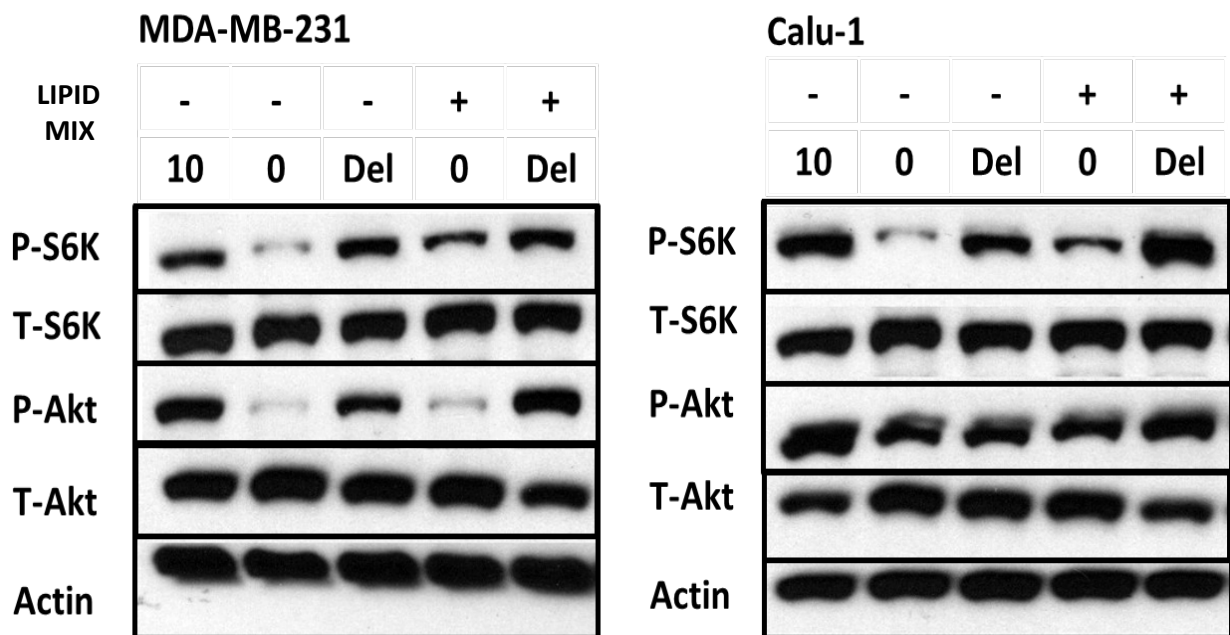


Figure 3.1 Lipid mix stimulates mTORC1 and mTORC2 activity.

MDA-MB-231 and Calu-1 cells were plated at 60% confluence and serum deprived (0 % serum) or shifted to delipidated (del) serum for 16hrs. 1:50 dilution of the lipid mix: BSA conjugate was added for 30 minutes. Cells were collected and analyzed for protein expression by western blot.

Lipid removal was achieved by either serum deprivation or shifting the cells to delipidated medium (reduced lipid content). As seen in Figure 3.1, there occurs a decrease in mTORC1 and mTORC2 activity upon removal of lipids as is evidenced by decrease in phosphorylation of S6K (mTORC1 substrate) and Akt (mTORC2 substrate) in MDA-MB-231 and Calu-1 cells. Addition of commercially available lipid mix (Table 3.1) caused an increase in mTORC1 and mTORC2 activity (Figure 3.1). The effect on mTORC2 activity as evidenced by increase in phosphorylation of Akt is more pronounced.

Components	Concentration (mg/L)
Arachidonic Acid	2.0
Cholesterol	220.0
DL-alpha-Tocopherol Acetate	70.0
Ethyl Alcohol 100%	confidential
Linoleic Acid	10.0
Linolenic Acid	10.0
Myristic Acid	10.0
Oleic Acid	10.0
Palmitic Acid	10.0
Palmitoleic Acid	10.0
Pluronic F-68	90000.0
Stearic Acid	10.0
Tween 80®	2200.0

Table 3.1: Composition of commercially available lipid mix (chemically defined lipid concentrate).

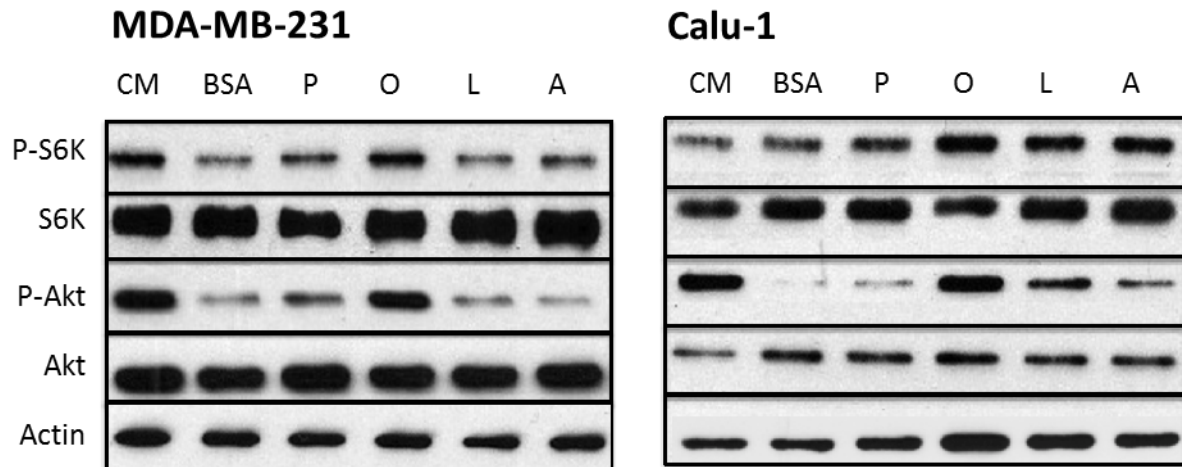


Figure 3.2 Exogenously Supplied Unsaturated Fatty Acids Stimulate mTORC1 and mTORC2 Activity.

MDA-MB-231 and Calu-1 cells were plated at 60% confluence in complete medium (CM) and shifted to serum free medium for 16 hr. Fatty acid (10 μ M): BSA in 2:1 ratio was added for 30 minutes. Fatty acid free BSA was used as a negative control. Lysates were collected and analyzed for phosphorylated mTOR substrates P-S6KT389 (mTORC1) and P-AktS473 (mTORC2) by Western blot. P, palmitic acid; O, oleic acid; L, linoleic acid; A, arachidonic acid. Western blots shown are representative of experiments repeated at least three times.

We examined the ability of different classes of fatty acids - Saturated fatty acid (Palmitate) and unsaturated fatty acids (Oleate, Linoleate, Arachidonate) to activate mTOR. Fatty acids were added in complex with Bovine Serum Albumin (BSA) in the absence of serum. As seen in Figure 3.2, the unsaturated oleic acid caused a robust increase in mTORC1 and mTORC2 activity as indicated by an increase in the phosphorylation of ribosomal subunit S6 kinase (S6K) at Thr389 (mTORC1) and Akt at Ser473 (mTORC2). We also observed a modest increase in mTOR activity with addition of linoleic acid in MDA-MB-231 cells; whereas, palmitic and arachidonic acid did not increase either mTORC1 or mTORC2 activity.

3.2 Phosphorylation of S6K and Akt is Mediated by mTORC1 and mTORC2 Respectively

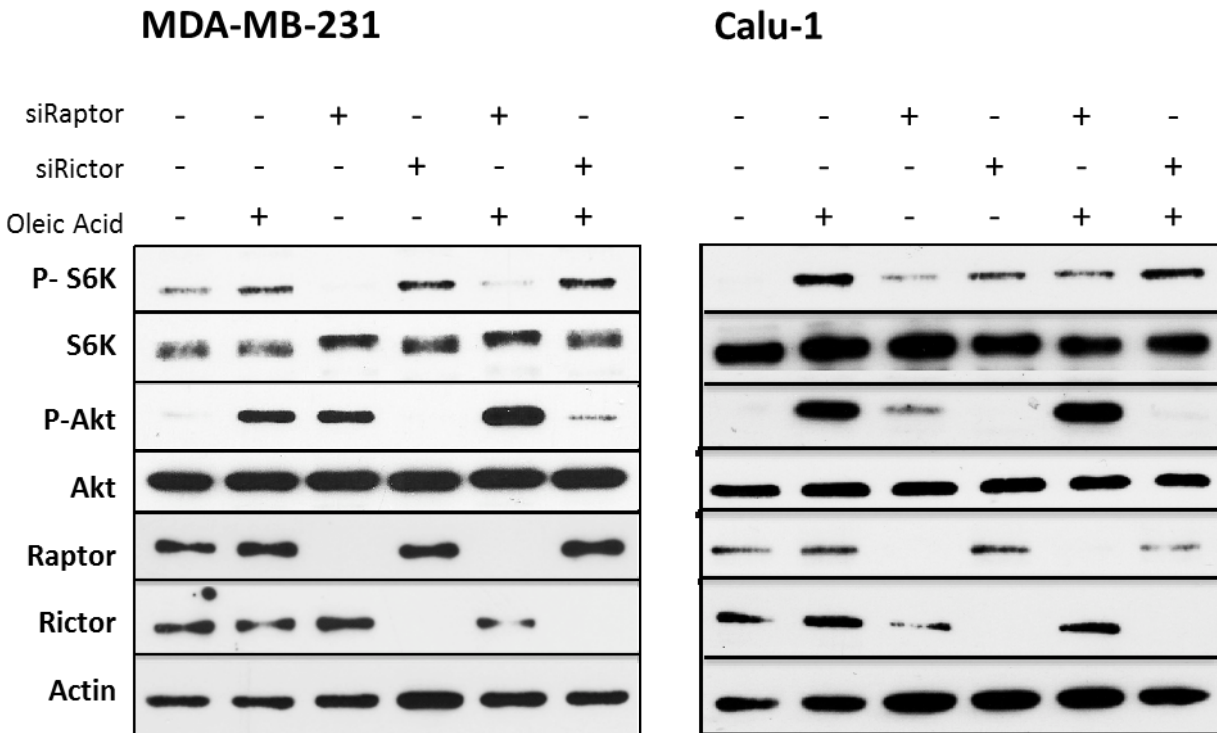


Figure 3.3: siRNA mediated knockdown of Raptor and Rictor abrogates oleic acid stimulation of mTORC1 and mTORC2.

MDA-MB-231 and Calu-1 cells were plated at 40% confluence and transfected with siRNA against Raptor (100 nM) and Rictor (100 nM). Cells were shifted to serum free media for the last 16 hr of transfection. Oleic acid (10 μ M): BSA in 2:1 ratio was added for 30 minutes. 72 hr post transfection cells were collected and lysates were analyzed for phosphorylated mTOR substrates by Western blot.

In an attempt to confirm requirement of mTORC1 and mTORC2 in activation of its substrates S6K and Akt respectively we looked at the oleic acid stimulation in absence of their binding partners. siRNA mediated knockdown of mTOR binding partners Raptor and Rictor abrogated the oleic acid- mediated increase in mTORC1 and mTORC2 activity respectively in MDA-MB-231 cells and Calu-1 cells (Figure 3.3). The knockdown Raptor induced phosphorylation Akt – consistent with the feedback activation of mTORC2 reported previously in response to inhibition of mTORC1 (O'Reilly et al., 2006). Taken together, exogenous unsaturated lipid Oleate provides a mitogenic signal to activate mTORC1 and mTORC2 in absence of any other serum factors.

3.3 Oleic Acid Mediated Activation of mTOR Occurs Through *De Novo* PA Synthesis

PA, which is required for mTOR complex formation and activity (Fang et al., 2001; Toschi et al., 2009), is also at the center of membrane phospholipid biosynthesis (Bonham et al., 2003). The negatively charged head group of PA binds to conserved positively charged amino acid residues on the FRB domain of mTOR (Foster, 2013; Veverka et al., 2008). Thus, the ability of oleic acid to stimulate mTOR suggests that activation of mTOR is via *de novo* synthesis of PA. A critical step in the synthesis of PA is the acylation of lysophosphatidic acid (LPA) by LPA Acyltransferase- β (LPAAT- β) (Figure 3.5, A). LPAAT- β also has a preference for unsaturated fatty acids – especially oleic acid (Hollenback et al., 2006). Besides, LPAAT- β activity has also been implicated in mTOR activation and growth of pancreatic cancer cell lines (Blaskovich et al., 2013). We therefore examined whether exogenously supplied oleic acid could activate mTOR in the absence of LPAAT- β .

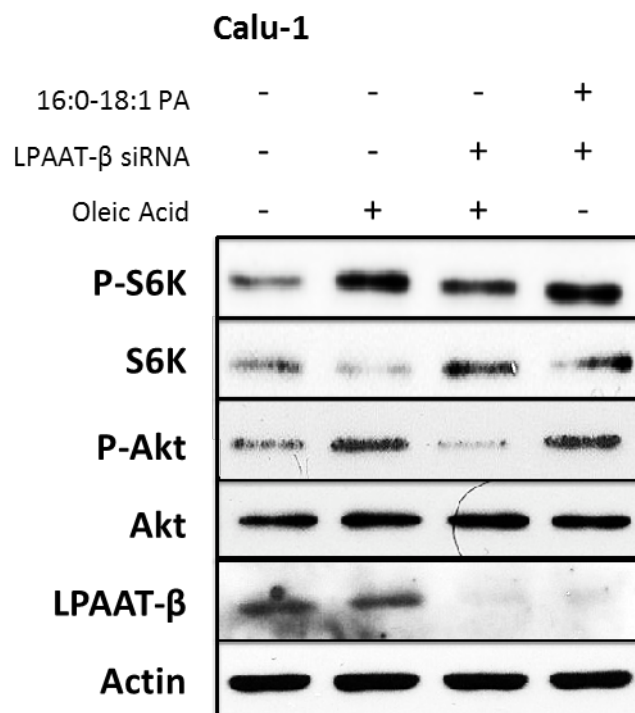


Figure 3.4: LPAAT- β inhibition prevents Oleic acid mediated activation of mTOR.

Calu-1 cells were plated at 40% confluence and transfected with LPAAT- β siRNA (100nM) or non-targeting siRNA for 48 hr. Cells were shifted to serum free media during the last 16 hr of treatment and 10 μ M oleic acid:BSA (2:1) was added to the cells for 30 minutes. Lysates were collected and probed for mTOR substrate phosphorylation and LPAAT- β by Western blot analysis.

As shown in Figure 3.4, knockdown of LPAAT- β with siRNA suppressed the oleic acid-induced activation of both mTORC1 and mTORC2 in Calu-1 cells – with a stronger impact on the mTORC2 substrate Akt. Importantly, the addition of 1-palmitoyl-2-oleoyl-PA (16:0, 18:1 PA), the product of LPAAT- β , could activate both mTORC1 and mTORC2 in Calu-1 cells where LPAAT- β expression was suppressed (Figure 3.4, Lane 4) – indicating that the suppressed mTOR activity observed with suppressed LPAAT- β expression was due to the lack of PA.

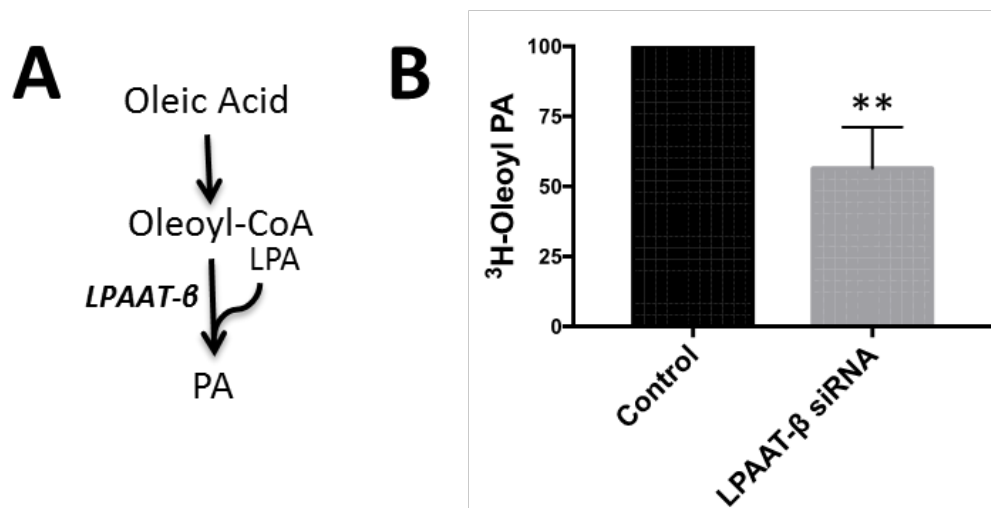


Figure 3.5: Inhibition of LPAAT-β decreases de novo PA production.

A) Schematic for the incorporation of oleic acid into PA. B) Calu-1 cells were plated at 40% confluence and transfected with LPAAT-β siRNA (100 nM) or non-targeting siRNA for 48 hr. During the last 16 hr, cells were shifted to serum free medium and [^3H]-oleic acid was added for 3 hr. Total cellular lipids were extracted and subjected to thin layer chromatography along with PA standard. PA levels were quantified using autoradiography. The statistical significance (p value) was determined by Student's two-tailed unpaired t test. **, $p \leq 0.01$ compared with the control (n=3).

Exogenously supplied oleic acid is activated to Oleoyl-CoA which can be shunted towards de novo PA synthesis by adding it to Lysophosphatidic acid (LPA) by LPAAT-β (Figure 3.5, A). We therefore examined the incorporation of [^3H]-oleic acid into PA with and without LPAAT-β expression. As shown in Figure 3.5, we observed a 50% reduction of [^3H]-oleic acid incorporation into PA in Calu-1 cells where LPAAT-β expression was suppressed. These data reveal that exogenously supplied oleic acid is incorporated into PA in an LPAAT-β-dependent manner and that activation of mTOR by oleic acid is dependent on LPAAT-β.

3.4 Acyl-CoA Synthetase Long Chain 5 Mediates mTOR Activity in KRas-Driven Cancer Cells

If the oleic acid is activating mTOR via the LPAAT- β catalyzed acylation of LPA, oleic acid needs to esterify with CoA. Fatty acids are esterified with CoA by a class of enzyme known as acyl-CoA synthetase (ACS). Of the many isoforms of ACS, ACS long chain-1 (ACSL1) and ACSL5 have been shown to have substrate specificity for unsaturated fatty acids (Mashek et al., 2007). Mutant KRas-driven cancers are known to scavenge for exogenous lipids (Jurre J Kamphorst, 2012; Salloum et al., 2014). It was reported that mutant KRas drives ACSL5 expression in HCT-116 colon cancer cells (Chun et al., 2010).

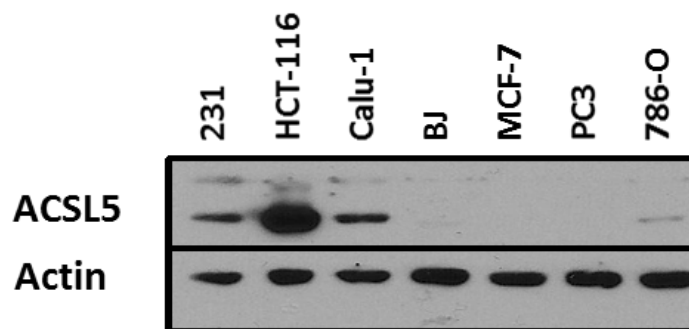


Figure 3.6: ACSL5 is expressed at high levels in mutant KRas cells.

Cell lysates from the indicated cancer cell lines were probed for expression of ACSL5 by Western blot.

We examined the level of ACSL5 expression in the KRas-driven HCT-116 colon cancer cells and the MDA-MB-231 and Calu1 cells used in Figures 3.2 and 3.4. We also examined the level of ACSL5 expression in several non-KRas-driven cancer cells (MCF7 breast, PC3 prostate, and 786-O renal) and the non-transformed human fibroblast cell line BJ-hTERT. As shown in Figure 3.6, the KRas-driven cancer cells express higher levels of ACSL5 expression relative to the non-KRas mutant cancer cell lines.

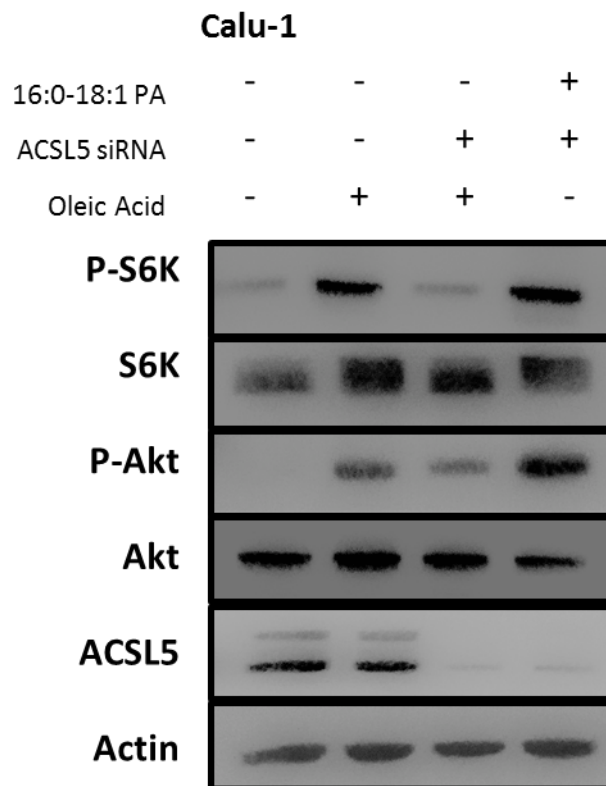


Figure 3.7: ACSL5 inhibition prevents Oleic acid mediated activation of mTOR.

Calu-1 cells were transfected with ACSL5 siRNA or non-targeting siRNA for 72 hr. Cells were shifted to serum free media during the last 16 hr of treatment and oleic acid (10 μ M): BSA (2:1) was added to the cells for 30 minutes and lysates were probed for the indicated proteins by Western blot.

To determine whether ACSL5 was required for the oleic acid-induced increases in mTOR activity, we performed siRNA knockdown of ACSL5. Knockdown of ACSL5 expression suppressed the oleic acid induction of both S6K and Akt phosphorylation (Figure 3.7). Exogenously provided 16:0, 18:1 PA was able to reverse the effect of ACSL5 knockdown on mTOR activity (Figure 3.7, lane 4).

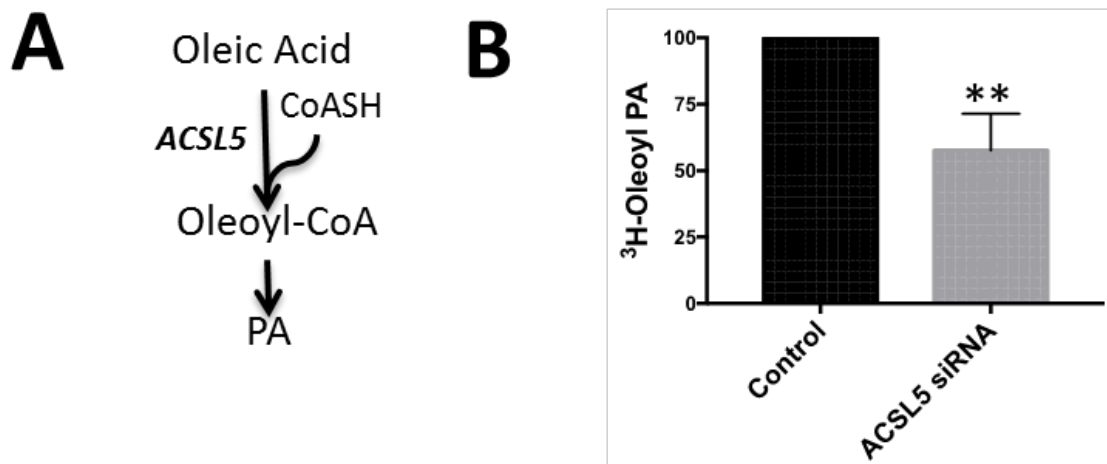


Figure 3.8: Inhibition of ACSL-5 decreases de novo PA production.

Calu-1 cells were transfected with ACSL5 siRNA or non-targeting siRNA for 72 hr. During the last 16 hr cells were shifted to serum free medium and [³H]-oleic acid was added for 3 hr. Total cellular lipids were extracted and subjected to thin layer chromatography along with PA standard. PA levels were quantified using autoradiography. The statistical significance (p value) was determined by Student's two-tailed unpaired t test. **, $p \leq 0.01$ compared with the control (n=3).

ACSL prepares fatty acids for phospholipid synthesis by addition of Coenzyme A in an ATP dependent reaction. Acyl-CoA synthetase adds Coenzyme A to fatty acids entering the cells shunts them to pathways of phospholipid metabolism (Figure 3.8). We tested the impact of ACSL5 knockdown on incorporation of oleic acid into PA. ACSL5 knockdown suppressed incorporation of [³H]-oleic acid into PA (Figure 3.8, B). The data in Figure 3.7 and 3.8 demonstrate that the oleic acid induction of mTOR is dependent on the generation of oleoyl-CoA.

3.5 Impact of ACSL5 Inhibition on Cell Cycle

The suppression of mTOR can cause the arrest of cells in G1 phase of the cell cycle (Fingar et al., 2004; Saqcena et al., 2013). We therefore examined the impact of suppressing ACSL5 on cell cycle progression in KRas-driven cancer cell line Calu-1. ACSL5 expression is elevated in KRas-driven cancer cells (Figure 3.6), which are known to scavenge lipids. Calu-1 cells were plated in complete medium with 10% fetal bovine serum as a source of lipids and TGF- β , which is required for cell cycle arrest caused by mTORC1 inhibition (Chatterjee et al., 2015; Gadir et al., 2008). Calu-1 cells were then treated with either scrambled or siRNA targeting ACSL5. After 96 hours, cells were harvested and subjected to flow cytometric analysis to determine the distribution of cells in G1, S and G2/M phase of the cell cycle. As shown in Figure 3.9 A, the percentage of cells in G1 increased, whereas the cells in S phase decreased. There was also a substantial reduction in incorporation of ³H-Thymidine into the DNA (Figure 3.9B).

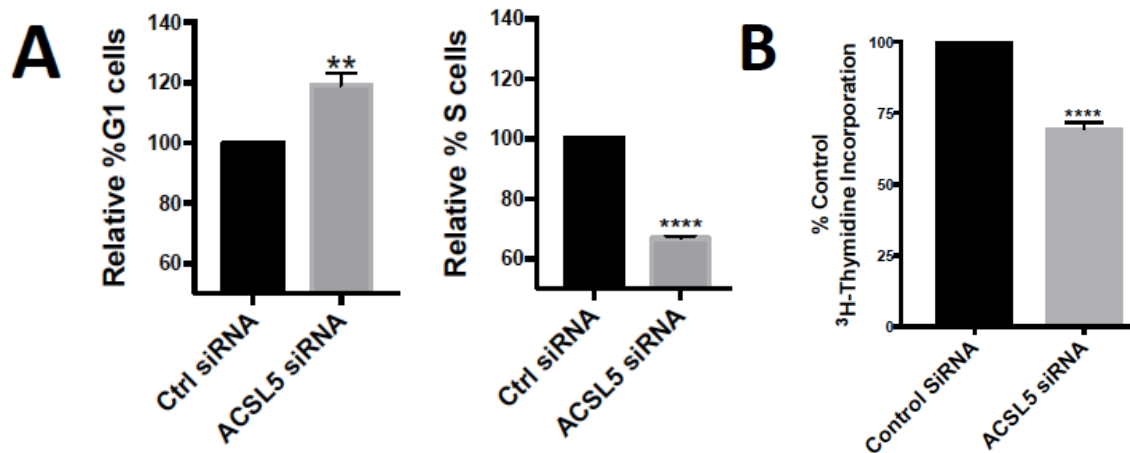


Figure 3.9: Suppression of Acyl-CoA Synthetase 5 expression leads to G1 cell cycle arrest.

(A) Calu-1 cells were plated at 40 % confluence and transfected with ACSL5 siRNA (25nM) or control siRNA. 96 hours post transfection cells were collected and analyzed by flow cytometry. Data from is shown in terms of percentage cells in each phase of cell cycle (n=3). (B) Calu-1 cells were plated at 40% confluence and transfected with ACSL5 siRNA or control siRNA. 48 hours post transfection ³H-Thymidine was added. After 24 hours cells were collected and total radioactivity was determined using scintillation counter. The statistical significance (p value) was determined by Student's two-tailed unpaired t test. **, $p \leq 0.01$; ****, $p \leq 0.0001$ compared with the control (n=3).

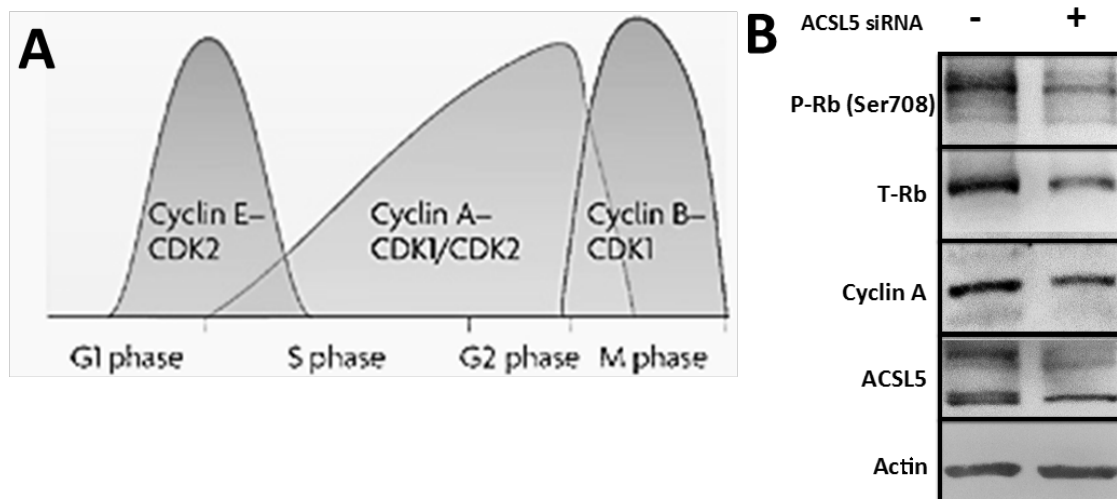


Figure 3.10: Suppression of ACSL5 leads to increased levels of G1 cell cycle markers.

(A) Changes in levels of Cyclins during different phases of cell cycle Adapted from (Hochegger et al., 2008) (B) Calu-1 cells were plated at 40% confluency and transfected with ACSL5siRNA (25nM) or control siRNA. 96 hours post transfection cells were collected and analyzed using western blot.

We also looked at the cell cycle markers. We observed a decrease in Rb, and the S phase cyclin (cyclin A) Figure 3.10. These data demonstrate that suppression of the pathway for lipid scavenging that ultimately gets incorporated into PA results in block of cell cycle progression, thereby highlighting the significance of this pathway in cell proliferation.

3.6 Cytosolic G3P Dehydrogenase-1 Generates the G3P Backbone Required for *De Novo* PA Synthesis and mTOR Activation Stimulated By Oleic Acid

To generate PA from oleoyl-CoA, a G3P backbone is needed for acylation. The major source of G3P is the glycolytic intermediate DHAP, which is reduced by cytosolic NAD-linked G3P dehydrogenase 1 (GPD1). GPD1 isotope tracing experiments were performed with uniformly labeled [^{13}C]-glucose to determine whether DHAP is being shunted away from glycolysis to G3P (see Figure 3.11A)

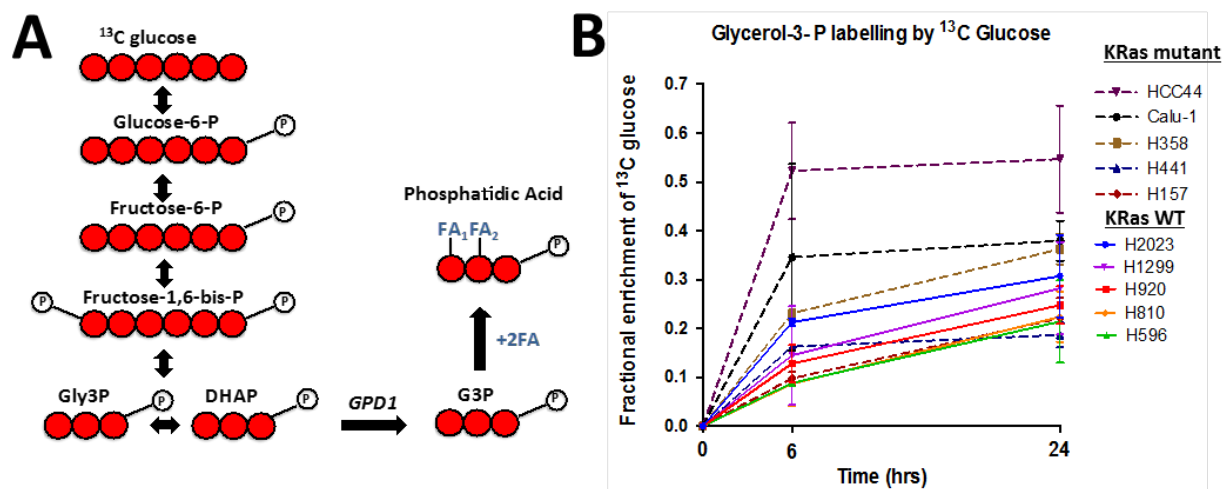


Figure 3.11: Glycolytic intermediate Dihydroxy acetone phosphate is shunted to generate Glycerol-3-Phosphate backbone for PA.

A) Model for tracing [^{13}C]-glucose carbon labeling to generate G3P and PA. B) Stable isotope tracing and GC-MS was used to measure fractional enrichment of [^{13}C]-glucose into m+3 G3P at 6 and 24 hr in a set of wild type and mutant KRas non-small cell lung cancer cell lines grown in complete medium.

We employed a panel of non-small cell lung cancer cell lines that were both KRas-driven and KRas wild type. [^{13}C]-glucose was added to the cells and the fraction of G3P labeled with ^{13}C was determined at 6 and 24 hr. As shown in Figure 3.11(B), all of the cells displayed substantial [^{13}C]-labeled G3P – indicating that some glucose-derived DHAP was being shunted to G3P. While the KRas mutant cell lines Calu-1 and HCC44 showed the largest fractional enrichment of glucose into G3P, there was not a complete correlation between KRas-driven cancer cell lines and higher percentage of [^{13}C]-labeled G3P. However, it was clear that substantial G3P was being generated from glucose.

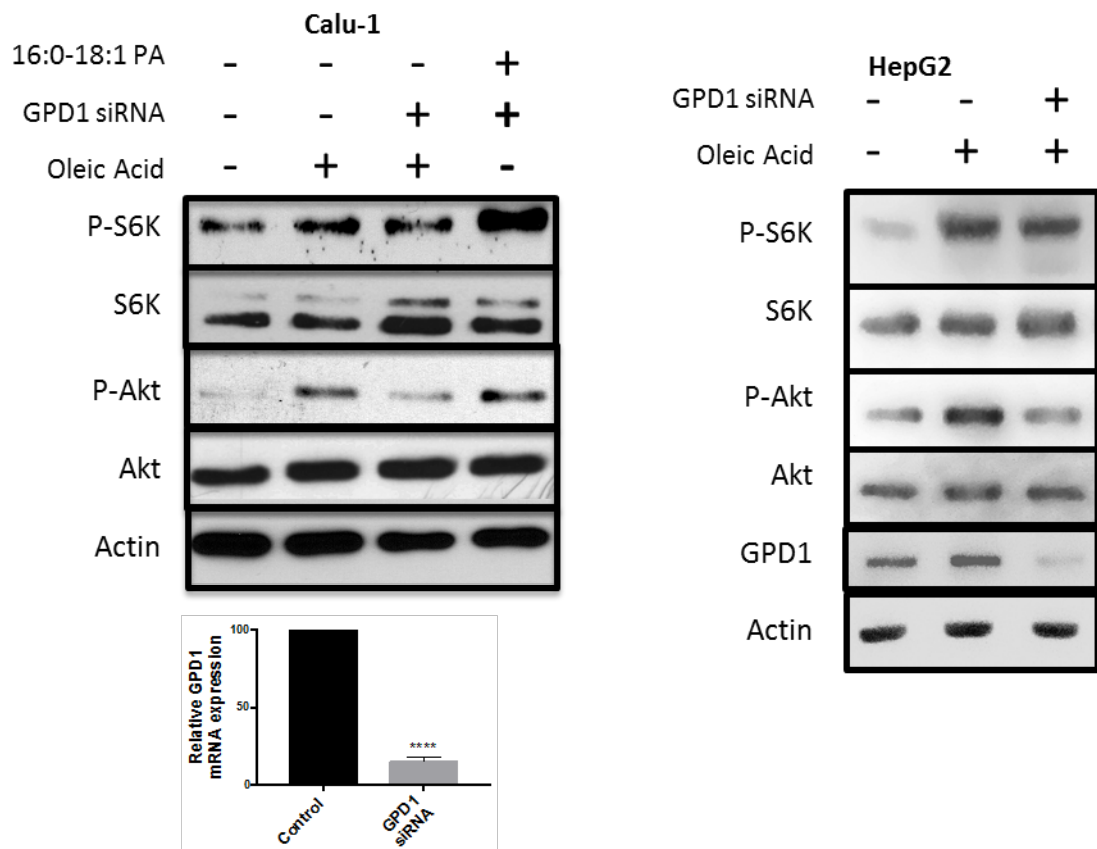


Figure 3.12: Inhibition of GPD-1 prevents Oleic acid mediated activation of mTORC1 and mTORC2.

Calu-1 and Hep-G2 cells were transfected with GPD1 or non-targeting siRNA for 48 hr. Cells were shifted to serum free media during the last 16 hr of treatment and 10 μ M Oleic acid:BSA (2:1) was added to the cells for 30 minutes. Lysates were probed for the phosphorylated mTOR substrates S6K and Akt by Western blot analysis as in Figure 3.2. GPD1 mRNA levels were determined for the Calu-1 cells and GPD1 protein levels were determined for the Hep-G2 cells.

We next examined the effect of suppressing the expression GPD1 – the enzyme that generates G3P from DHAP on oleic acid-induced phosphorylation of S6K and Akt in Calu-1 cells. As shown in Figure 3.12, treatment with GPD1 siRNA suppressed mTORC1 and mTORC2 activation as indicated by reduced phosphorylation of S6K and Akt. Exogenously provided 16:0, 18:1 PA was able to stimulate both S6K and Akt phosphorylation when GPD1 expression was suppressed. For reasons that are not clear, the levels of GPD1 protein are very low in Calu-1 cells making it difficult to establish that knockdown of GPD1 was actually occurring in response to GPD1 siRNA. However, we could detect strong reduction in the level of GPD1 RNA levels (Figure 3.12,A). To further establish a dependence of oleic acid-induced mTOR activation on GPD1, we examined the effect of GPD1 knockdown in the N-Ras-driven HepG2 hepatoma cells (Omerovic et al., 2008) that are known to express detectable levels GPD1 (Uhlen et al., 2015). As shown in Figure 3.12, knockdown of GPD1 suppressed the oleic acid-induced activation of both mTORC1 and mTORC2; and with the HepG2 cells, we could see reduction in GPD1 protein.

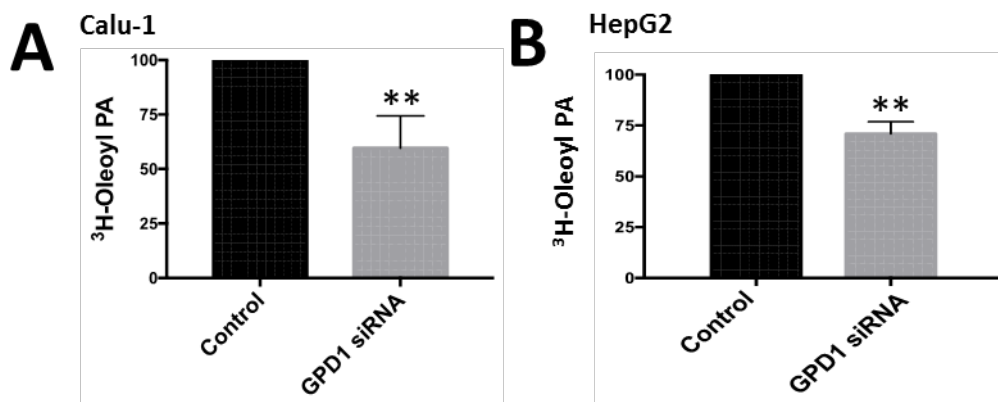


Figure 3.13: Inhibition of GPD1 decreases *de novo* PA production.

Calu-1 and HepG2 cells were transfected with GPD1 or non-targeting siRNA for 48 hr. Cells were shifted to serum free media for the last 16 hr of treatment and [^3H]-oleic acid was added for 3 hr. Total lipids were extracted and radiolabeled PA was determined. The statistical significance (p value) was determined by Student's two-tailed unpaired t test. **, $p \leq 0.01$ compared with the control (n=3).

We next determined whether the [^3H]-oleic acid incorporation into PA was dependent on GPD1. The Calu-1 and HepG2 cells were treated with scrambled or GPD1 siRNA for 48 hr. [^3H]-oleic acid was added for the final 3 hr. Radiolabeled PA was determined as in Figures 3.5 and 3.8. As shown in Figure 3.13(A) (Calu-1 cells) and Figure 3.13(B) (HepG2 cells), the level of [^3H]-labeled PA was significantly reduced by knockdown of GPD1. Collectively, the data demonstrate that the oleic acid induction of mTOR is dependent on glucose-derived G3P and GPD1.

The data provided here demonstrate that mTOR responds to exogenously supplied fatty acids via the *de novo* synthesis of PA (Figure 3.14).

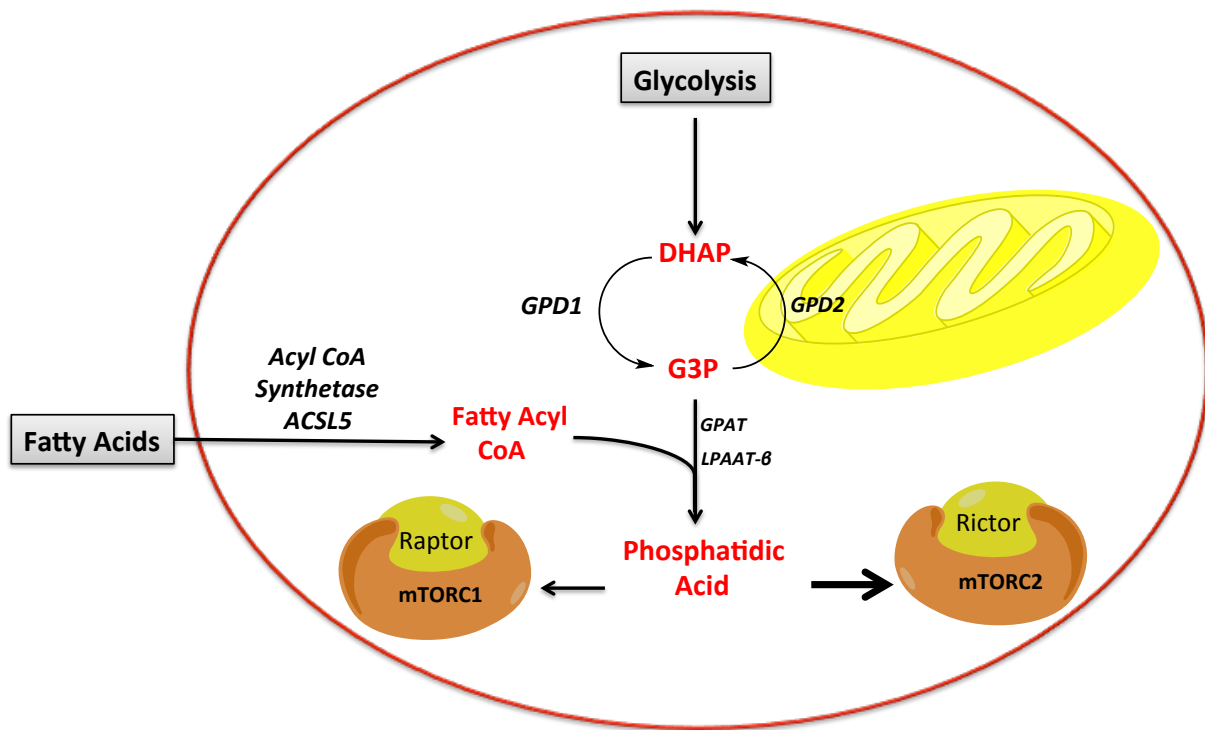


Figure 3.14: Schematic for mTOR Activation in Response to Fatty Acids.

Exogenously supplied fatty acids stimulate mTOR via *de novo* PA synthesis. The glycerol backbone of PA comes from reduction of the glycolytic intermediate DHAP by GPD1. CoA is acylated with fatty acids by ACSL5 to generate fatty acyl-CoA. The fatty acid can then be transferred to the glycerol backbone of G3P by a glycerophosphate acyltransferase (GPAT) to generate LPA, which can then be acylated by LPAAT. The responsiveness of mTOR to lipids and glucose via the generation of PA represents a means for the sensing critical nutrients for cell growth.

Localization of mTORC2 Upon Lipid Sensing

An interesting aspect of mTOR activation in response to oleic acid is that mTORC2 is more responsive to oleic acid than is mTORC1. Akt gets phosphorylated at the mTORC2 site at Ser473 more strongly than S6K gets phosphorylated at the mTORC1 site at Thr389 (Figure 3.2). This could be due to subcellular localization. While mTORC2 has been reported to have several sub-cellular locations, it was recently reported that mTORC2 co-localizes with mitochondrial associated endoplasmic reticulum membranes (MAMs) (Betz et al., 2013). The MAMs are extensions of the endoplasmic reticulum that represent a hub for control over cellular metabolism (Vance, 2014). MAMs are also enriched in lipid metabolism enzymes including LPAAT (Yamashita et al., 2014) and ACSL5 (Mashek et al., 2006). Thus, the more profound effect of oleic acid on mTORC2 may be due to the synthesis of PA on the MAMs where mTORC2 localizes. It is also of interest that the mTORC2 substrate Akt also localizes at the MAMs (Boulbes et al., 2011). mTORC2 has been implicated in regulating metabolism and mitochondrial functions ■ thus, the responsiveness of mTORC2 to PA levels may be a reflection of PA synthesis via the *de novo* biosynthetic pathway that is taking place on MAMs.

3.7 mTORC2 Activation Leads to Phosphorylation of ATP Citrate Lyase

Cancer cells shift to an anabolic mode of metabolism to sustain high rates of proliferation. *De novo* lipogenesis is a key anabolic pathway that generates sufficient fatty acids for membrane biosynthesis (Beloribi-Djefalia et al., 2016). The first step in synthesis of *de novo* fatty acids in the cell is conversion of citrate to Acetyl CoA, catalyzed by ACL (Figure 1.9). Acetyl CoA generated by this step can be used for *de novo* fatty acid synthesis to generate Palmitic acid. Activation of ACL involves phosphorylation of ACL by active mTORC2 substrate Akt (Berwick et al., 2002). As seen in Figure 3.2, upon addition of oleic acid, the impact on phosphorylation of Akt (mTORC2 substrate) is much stronger than P-S6K (mTORC1 substrate). Therefore we decided to look if activation of mTORC2 by oleic acid had an impact on phosphorylation of ACL and thus *de novo* lipogenesis. HCT-116 cells were serum starved and oleic acid was added to look at the impact on activation of ACL. Addition of oleic acid leads to phosphorylation of ACL at S454 by Akt (mTORC2) substrate (Figure 3.16). This effect was similar to as seen upon insulin stimulation that is known to activate mTORC2 and lipid synthesis in cells (Saltiel and Kahn, 2001). In order to confirm the requirement of mTORC2 we used Torin1, a competitive mTOR kinase inhibitor. Addition of Torin1 completely blocked phosphorylation of Akt at S473 and ACL at S454 upon addition of oleic acid.

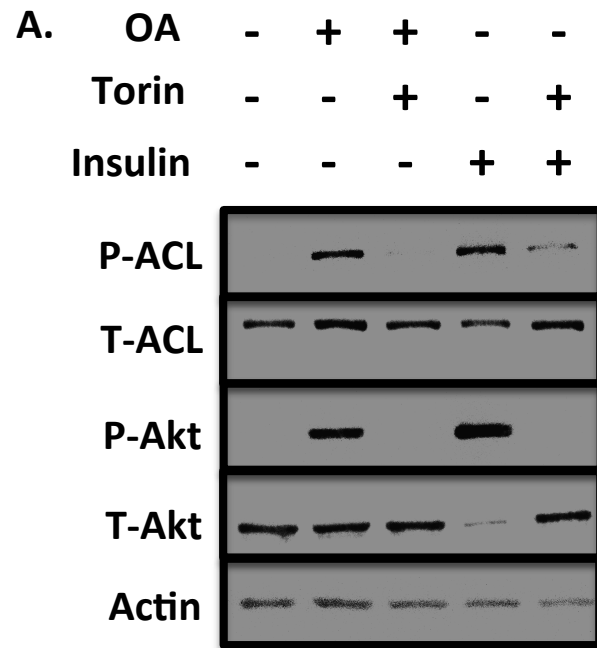


Figure 3.15: Oleic Acid stimulates phosphorylation and activation of ATP Citrate Lyase in a mTORC2 dependent manner.

A) HCT-116 cells were plated at 60% confluence and deprived of serum overnight. Oleic acid (30 μ M) was added in complex with BSA (2:1) in presence or absence of Torin1 (1 μ M). Insulin (100nM) was added in the indicated wells. Lysates were collected and analyzed for protein expression for mTORC2 target P-Akt (S473) and P-ACL (S454) by western blot.

3.8 mTORC2 Translocates to Mitochondrial Fraction Upon Activation by Oleic Acid.

Since Akt phosphorylation at Ser473 is catalyzed by mTORC2 (Sarbasov et al., 2005), this observation suggests that mTORC2 responds more robustly to lipids than mTORC1. mTORC2 has been reported to localize to the endoplasmic reticulum (ER) (Boulbes et al., 2011), and more specifically, to mitochondrial associated ER membranes (MAMs) where it plays an important role in mitochondrial physiology (Betz et al., 2013). It was proposed that the MAMs are an mTORC2 signaling hub (Betz et al., 2013). We isolated a mitochondrial cell fraction and examined changes in the subcellular localization of mTOR and Rictor upon addition of oleic acid. We also examined the presence of the MAM marker acetyl CoA synthetase long chain 4 (ACSL4) and the mitochondrial marker – voltage dependent anion channel 1. As shown in Figure 3.16, both mTOR and Rictor migrate to mitochondrial fraction in response to oleic acid. There was a corresponding loss of mTOR and Rictor from the cytosolic fraction when cells were treated with oleic acid. The association of Rictor with the mitochondrial fraction is most likely due to an oleic acid-induced association of mTORC2 with MAMs as was reported recently by Michael Hall's group (Betz et al., 2013). Our data suggests that mTORC2 can be activated on MAMs in response to oleic acid. LPAAT and ACSL5 enzymes also localize to the mitochondrial and ER membranes. Thus, there is a possible role of *de novo* PA in the localization of mTORC2 to the mitochondrial fraction.

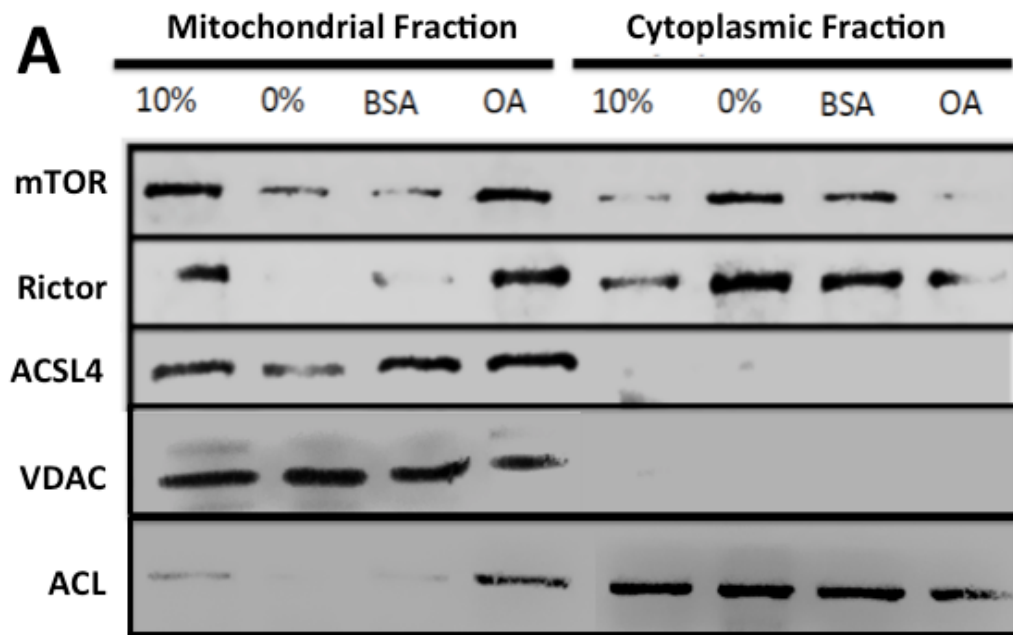


Figure 3.16: mTORC2 and ACL associate with MAMs in response to oleic acid.

HCT-116 cells were plated at 60% confluence and deprived of serum overnight. Oleic acid (30uM): BSA (2:1) was added to the cells for 30 minutes. Mitochondrial fractions separated from cytoplasmic fractions and probed for protein expression by Western blot. 10%- Complete media; 0%- serum deprived media; BSA- Bovine serum Albumin; OA-Oleic acid.

3.9 Summary of Results

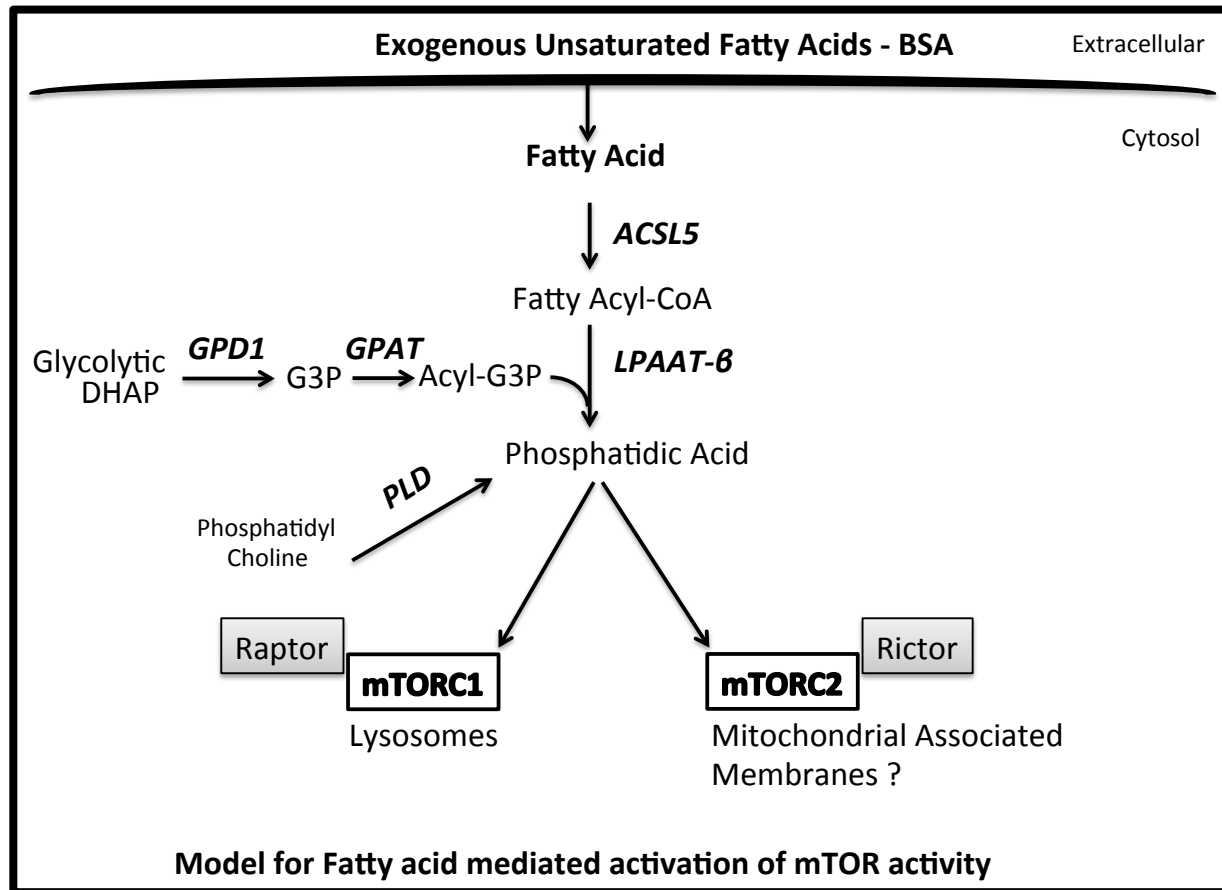


Figure 3.17: Figure summarizing findings of the study.

mTOR responds to exogenously supplied fatty acids via synthesis of de novo Phosphatidic acid. Exogenously supplied oleic acid in complex with BSA enters the cell and is activated by ACSL5. Activated Oleoyl-CoA is shunted towards de novo PA acid synthesis required for mTORC1 and mTORC2 activity. mTORC2 in response to oleic acid addition localizes to the mitochondrial fraction.

- Exogenous unsaturated fatty acid (Oleate) activates mTORC1 and mTORC2
- Activation of mTOR by Oleate is dependent on LPAAT- β generated Phosphatidic Acid.
- Ras – driven cancer cells that depend on exogenous lipids for survival, express higher levels of ACSL5 that activates lipids entering into cell shunting it towards anabolic phospholipid synthesis.
- The activation of mTORC1 by oleic acid is dependent upon glucose-derived DHAP.
- *De novo* lipid sensing by mTOR drives mTORC2 to the mitochondrial fraction, whereby *de novo* lipid synthesizing enzymes are localized.
- Activation of mTORC2 in response to exogenous lipids drives ACL to mitochondria and thus initiates *de novo* lipogenesis.
- The PA-dependency of mTOR represents a mechanism for sensing lipids and glucose (Figure 3.17).

Chapter 4 DISCUSSION AND FUTURE DIRECTIONS

4.1 Lipid Mediated Activation of mTOR

Lipids are an essential component of the cell membranes and also contribute to cellular signaling. The role of lipids as transcription factors is of particular significance (Jump, 2004). Despite advances in understanding the chemistry of lipids and lipid synthesis pathways, the process of how cells sense dietary lipids is not well understood. Lipid deprivation arrests cells in G1 phase of the cell cycle (Patel et al., 2016), but it is unclear how cells sense lipid sufficiency in order to proliferate. mTOR is a key regulator of cellular growth and proliferation, activation of which allows cells to progress from G1 to S phase of cell cycle (Fingar et al., 2004). While much is known about the amino acid input to mTORC1 on lysosomal membranes (Efeyan et al., 2012) there is very little known about the glucose input to mTOR and nothing about any input to mTOR involving lipids.

In this dissertation, we provide evidence that lipids impact both mTORC1 and mTORC2 via the *de novo* synthesis of PA – a central metabolite for membrane phospholipid biosynthesis. There is a requirement for both fatty acids and G3P, a product of glycolysis, for the activation of mTOR. Thus, the PA needed for mTOR activation reflects the presence of both lipids and glucose. Our data demonstrates that the nutrient sensing by mTOR goes beyond that for amino acids and includes input from both lipids and glucose via the production of PA.

A previous study made an interesting observation that PA moiety with two saturated fatty acids (di-palmitoyl PA) inhibits mTORC2 activity (Zhang et al., 2012). Our work demonstrates that PA with one saturated and unsaturated fatty acid (1-palmitoyl-2-oleoyl-PA) activates both mTORC1 and mTORC2. The difference can be partially attributed to structural differences of PA species. The presence of an unsaturated lipid in PA promotes formation of an inverted cone

shaped phospholipid structure (Scales and Scheller, 1999; Sung et al., 2003), which could possibly allow for insertion of the PA head group into the hydrophobic pocket of the mTOR-FRB domain (Figure 1.7). This hypothesis of differential binding of diverse PA species to FRB domains requires further structural investigation. Co-immunoprecipitation of mTOR complexes and using mass spectrometry to analyze the associated phospholipid species will throw light on the activator and inhibitory roles of PA. Another interesting line of investigation would be to study the nature and composition of acyl chains in the PA species generated by different enzymes (PLD, DGK and LPAAT) based on substrate specificities. We hypothesize that cells regulate mTOR activity through generation of different PA species.

4.2 What Drives mTOR to its Destination: Localization of mTOR

PA binds to mTOR at conserved residues in the hydrophobic pocket of FRB domain through its interaction with positively charged Arg residue (Fang et al., 2001; Veverka et al., 2008; Wiczer and Thomas, 2012). Upon amino acid stimulation, PLD translocates to the lysosome in a Vps34 dependent manner (Yoon et al., 2011). PLD generated PA mediates amino acid and glucose sensing by mTORC1 (Xu et al., 2011) and has been shown to be required for localization of mTORC1 to the lysosomes (Wiczer and Thomas, 2012). mTORC1 is known to associate with Rag proteins that are essential for its activation at the lysosomes. Therefore it can be postulated that PA is essential but not sufficient to drive mTOR activity. However, the exact mechanism as to how PLD generated PA interacts with mTOR to lysosome is still unknown. One possibility is that PLD localized at the lysosomal membrane generates PA enriched domains that allow FRB domain to interact and dock to the lysosomes, in association with the Rag proteins that are required for its activation (Sancak et al., 2010).

mTORC2 was recently reported to be localized to MAMs, which are at the interface of mitochondria and the ER (Betz et al., 2013). By localizing to the MAMs mTORC2 regulates mitochondrial physiology and function. In our studies we observe that mTORC2 is activated and localizes to mitochondrial fraction in response to exogenous unsaturated lipids (Figure 3.16). In this report, we have shown that mTORC2 activity requires synthesis of *de novo* PA generated by LPAAT- β (Figure 3.4). LPAAT- β is known to be associated with the ER membrane (Takeuchi and Reue, 2009). It is speculated that LPAAT- β generated PA plays a role in recruitment of mTORC2 to the MAMs and is an area of future exploration. In this regard we have generated LPAAT- β knockout HCT-116 cells and are currently exploring the requirement of *de novo* PA for the membrane localization of mTORC2 in response to oleic acid.

4.3 Role of PA as A pH Sensor During mTOR Activation

As PA has a pK_a between 6.9-7.9, at physiological pH, PA has a negatively charged head group (Shin and Loewen, 2011). Simons et al. elegantly showed the role of PA as a pH sensor in regulating the binding of Dvi protein to membranes, crucial for activation of Wnt signaling pathway (Simons et al., 2009). NHE2 pumps protons across the plasma membrane, creating a basic environment in the cytosol, that results in increase of net negative charge on PA head group. The negative charges on PA interact with basic residues on Dvi protein thus establishing the role of PA as a pH sensor. siRNA mediated knockdown of NHE2 exchanger prevents binding of Dvi protein to peripheral membrane thus blocking the Wnt signaling pathway.

We speculate such a similar pH dependent interaction promotes assembly of mTORC1 and mTORC2 complex at the lysosomal membrane and MAMs respectively. Lysosomal membrane is associated with V-type ATPase pumps that move protons into the lysosome, allowing for acidification of the lysosomal lumen (Mindell, 2012). Movement of protons across the V-type ATPase creates a localized basic environment outside the lysosome whereby negatively charged PA in the membrane can promote localization of mTOR with the lysosomal membrane.

Along the same lines, association of mTORC2 complex with the mitochondria might be promoted through a basic environment created by transport of ions across the Voltage dependent anion channel that helps maintain a membrane potential across the cytosol and mitochondrial inter-membrane space. This would allow for more negatively charged PA to interact with the FRB domain of mTOR.

4.4 Lipid Sensing and KRas-Driven Cancers

The bulk of this study was performed using KRas-driven cancer cells which have an enhanced need for exogenous lipids (Salloum et al., 2014, Kamphorst et al., 2013). The work detailed in the dissertation demonstrates that mTOR responds to exogenously supplied fatty acids via the *de novo* synthesis of PA. It is of interest that LPAAT- β silencing was able to inhibit the proliferation and anchorage-independent growth of pancreatic cancer cells (Blaskovich et al., 2013) – wherein KRas is mutated in 95% of the cases (Bryant et al., 2014). Inhibition of LPAAT- β was shown to have a synthetic lethal effect in combination with mutant KRas in shRNA-based screen (Luo et al., 2009). Thus, the *de novo* PA biosynthesis pathway appears to be critical for the proliferation and survival of cancer cells that are dependent on mutant KRas signals. The importance of this pathway in KRas-driven cancers makes the targeting the PA synthesis pathway in the large number of KRas-driven human cancers, an interesting therapeutic opportunity.

Mammalian cells synthesize palmitic acid that can be elongated and then de-saturated by SCD1 to generate oleic acid. KRas-driven cells lack expression of SCD1, and depend on exogenously supplied unsaturated lipids (Salloum et al., 2014). Further, we observe an increased expression of ACSL5 in KRas-driven tumors. ACSL5 is responsible for catalyzing the addition of CoA to incoming unsaturated fatty acids and allows for its utilization in phospholipid biosynthesis. It has been previously shown that overexpression of ACSL leads to an increase in Acyl-CoA activity and an increase in exogenous lipid uptake (Mashek et al., 2006). Increased expression of ACSL5 could possibly allow for more exogenous fatty acids to enter the cell. This dependence of Ras driven cells on ACSL for utilization of exogenous lipids to survive, could

possibly be therapeutically exploited. Thus, ACSL5 inhibition could be a potential therapeutic target for KRas-driven tumors.

4.5 Targeting Alternative Sources of PA As A Therapeutic Target

PA shares a similar binding site on the FRB domain with that of mTOR inhibitor - rapamycin. PLD activity that is elevated in serum-deprived cells generates PA that has been shown to compete with rapamycin. Inhibition of PLD in cancer cells led to a decrease in PA levels, making it more sensitive to lower doses of rapamycin, which competes with PA for mTOR binding (Chen et al., 2003).

Our data shows that oleic acid mediated mTORC2 activation is dependent on LPAAT-generated PA. Therefore we speculate that PA generated by LPAAT- β can compete with rapamycin binding to mTORC2. It would be interesting to see if LPAAT- β inhibition increases the sensitivity of mTORC2 to Rapamycin treatment. Likewise, we have seen that inhibition of lipid uptake by using a macropinocytosis inhibitor ethylisopropyl amiloride, sensitizes cancer cells to rapamycin mediated cell death (Salloum et al., 2014).

GPD1 catalyzes the reduction of DHAP to G3P to form the backbone of *de novo* PA. Therefore GPD1 acts as a link between glycolysis and phospholipid synthesis. We see that the inhibition of GPD1 in Calu1 and HepG2 cells inhibits oleic acid mediated mTOR activation and incorporation of exogenously supplied oleate into PA (Figure 3.12). In order to proliferate, cancer cells need to synthesize PA for membrane phospholipid synthesis and mTOR activation. Interestingly GPD1 enzyme activity is overexpressed in bladder cancer and patients with

colorectal adenocarcinomas (Krasnov et al., 2009; Turyn et al., 2003) making GPD1 an unexplored target in cancer therapy.

Further, our study focused on *de novo* source of PA production through LPAAT. Preliminary data from our lab points to compensatory PA production upon inhibition of PLD activity. We see an increased incorporation of exogenous lipids into PA through LPAAT- β upon inhibition of PLD activity (Figure 4.1).

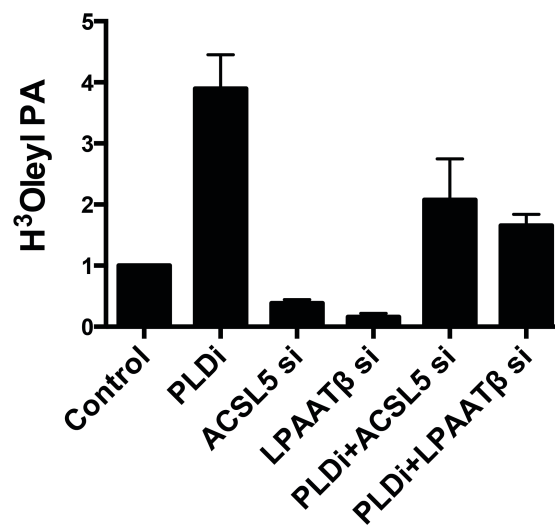


Figure 4.1: PLD inhibition leads to compensatory oleate incorporation into PA.

Calu-1 cells were transfected with ACSL5 siRNA or LPAAT- β siRNA for 48 hr. Cells were shifted to serum free media for the last 16 hrs. of treatment and [³H]-oleic acid was added for 3 hrs. along with PLD1 (10 μ M) and PLD2 (10 μ M) inhibitors in the indicated lanes.. Total lipids were extracted and radiolabeled PA was determined.

PLD null mice are viable and fertile, whereas mTOR knockout is embryonic lethal points to the significance of compensatory role played by other sources of PA (Gangloff et al., 2004) (Burkhardt et al., 2014). Therefore, we propose that PLD inhibitors in combination with LPAAT inhibition be more effective in inhibiting cellular growth and proliferation of cancer cells.

4.6 Summary

The data provided here demonstrate that: 1) exogenously provided lipids stimulate both mTORC1 and mTORC2 in an LPAAT and GPD1 dependent manner; and 2) that exogenously supplied lipids stimulate translocation of mTORC2 to MAMs along with ACL, which gets phosphorylated by Akt (mTORC2 substrate) at Ser454. The generation of PA via the Kennedy pathway in response to oleic acid promotes mTOR activation in KRas-driven cancer cells that rely on scavenging for obtaining both lipids (Kamphorst et al., 2013; Salloum et al., 2014) and proteins (Commisso et al., 2013). The significance of these data is that the PA requirement for mTOR likely represents a nutrient input to mTOR that reflects the presence of both lipids and glucose – and may be of greater significance in KRas-driven cancer cells where there is a greater dependence on exogenously supplied nutrients – which could represent a vulnerability for the many cancers driven by KRas.

Unanswered Questions

- Do different PA species generated through multiple pathways of PA generation lead to differential regulation of mTOR?
- Do pH differences across the lysosomal and mitochondrial membranes play a role in ability of PA to bind mTOR?
- Does *de novo* PA play a role in mTORC2 localization to the MAMs?
- Can *de novo* PA generated through LPAAT activity compensate for lack of PLD upon PLD inhibition?
- Are PLD null mice viable due to increased dependence on exogenous lipids and *de novo* PA generated through LPAAT?
- Is GPD1 activity elevated in KRas-driven cancers and potentially a viable therapeutic target?
- Is inhibition of ACSL5 lethal to KRas-driven tumors?

Chapter 5 REFERENCES

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