REGULATION OF NUTRIENT SIGNALING TO MAMMALIAN TARGET OF RAPAMYCIN BY PHOSPHOLIPASE D

by

Limei Xu

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

2012

This manuscript has been read and accepted for the Graduate

Faculty in Biology in satisfaction of the dissertation

requirement for the degree of Doctor of Philosophy.

10/13/2011	Dr. David Foster
Date	Chair of Examining Committee
10/13/2011	Dr. Laurel Eckhardt
Date	Executive Officer

Dr. Jill Bargonetti, Hunter College, CUNY

Dr. Diego Loayza, Hunter College, CUNY

Dr. David Solit, Memorial Sloan Kettering Cancer Institute

Dr. Hans-Guido Wendel, Memorial Sloan Kettering Cancer Institute

THE CITY UNIVERSITY OF NEW YORK

ABSTRACT

REGULATION OF NUTRIENT SIGNALING TO MAMMALIAN TARGET OF RAPAMYCIN BY PHOSPHOLIPASE D

by

Limei Xu

Adviser: Professor David A. Foster

The mammalian target of rapamycin (mTOR) is a key component of a complicated signaling network which transduces nutrient signals and many other stimuli to modulate a wide range of cellular functions, such as cell growth, cell proliferation and cell survival. Phospholipase D (PLD) is an enzyme which catalyzes the hydrolysis of phosphatidylcholine (PC) to form phosphatidic acid (PA). PA has been shown to be a very important lipid second messenger that mediates mitogenic signals upstream of mTOR and both PLD and mTOR have been implicated as cancer cell survival signals. Therefore it is of interest as to whether PLD plays a role in mTOR mediated nutrient signaling. We have found that elevated PLD activity in human cancer cells is dependent on the availability of both amino acids and glucose and that PLD is required for amino acid- and glucose-induced mTOR Complex 1 (mTORC1) activity. Moreover we investigated the possible regulators which are involved in mediating the nutrients signals including amino acids and glucose to PLD and mTORC1. We found that small GTPases RalA and ARF6 which form a complex with PLD to activate its activity are required for both PLD and mTORC1 activation induced by amino acids and glucose. The class III phosphatidylinositol-3-kinase hVps34 emerged as an important modulator for amino acid sensing. In this study, we showed that the depletion of hVps34 or

binding partner hVps15 with siRNA dramatically suppressed the PLD activity and further disrupted nutrient sensing to mTORC1, possibly by failing to recruit PLD to endomembrane and stimulate mTORC1 activity in response to nutrients. Taken together, these findings demonstrate that phosphatidic acid generated by PLD is a critical mediator that links nutrient signals to mTORC1, thus implicating the important role of PLD and PA in cancer cell proliferation and survival.

ACKNOWLEDGEMENTS

First, I want to express my sincere gratitude to my mentor Dr. David Foster for his continuous support, insightful discussion and comprehensive training during my dissertation research. His enthusiasm, his inspiration, and immense knowledge make cancer research more fun to me. Without his contributions of time, patience and guidance, it is impossible to finish the thesis.

Also I want to thank my colleagues from Dr. David Foster's lab supported me in my research work and my personal life. I want to thank them for all their helpful discussions, for their emotional support and fun moments they provided. I would also like to acknowledge Phil Medlin and Benjamin Perrella, two undergraduates with whom I've had the pleasure to work with.

I would like to thank my committee members Dr. Jill Bargonetti, Dr. Diego Loayza, Dr. David Solit, Dr. Hans-Guido Wendel for their time and service. In particular, I am grateful to Dr. Jill Bargonetti and Dr. Hualin Zhong for their great help during my lab rotation and my dissertation research. My sincere thanks also go to Dr. Lu-hai Wang for offering the research opportunity in his group at Mount Sinai and leading me to pursuing cancer research for my Ph.D. training.

Last, but most importantly, I want to thank my parents for their love and support throughout my life. I really appreciate my extended family for providing a caring and supportive environment for me. Especially, I would like to give my special thanks to my husband Jianqiang whose love and support made it possible to complete this thesis. I dedicate this thesis to all of you.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

LIST OF ABBREVIATIONS

AMPK: AMP-activated protein kinase

ARF: ADP ribosylation factor

DAG: diacylglycerol

FKBP12: FK506-binding protein 12

FRB: FKBP12–rapamycin binding domain

GAP: GTPase-activating protein

GDI: guanine nucleotide dissociation inhibitor

GEF: guanine nucleotide-exchange factor

mTOR: mammalian target of rapamycin

PA: phosphatidic acid

PBt: phosphatidylbutanol

PC: phophatidylcholine

PI3K: phosphoinositide 3-kinase

PIP_2: phosphatidylinositol-4,5-bisphosphate

PI3P: phosphatidylinositol 3-phosphate

PKC: protein kinase C

PLD: phospholipase D

PTEN: phosphatase and tensin homologue deleted on chromosome ten

$p70^{S6k}$: 70 kDa ribosomal protein S6 kinase

Raptor: regulatory associated protein of mTOR

Rheb: Ras homologue enriched in brain

TSC: tuberous sclerosis complex

CHAPTER I

INTRODUCTION

1.1 PHOSPHOLIPASE D

Our lab is interested in investigating signaling events in cancer cells, and discovered that Phospholipase D (PLD) activity is elevated in several types of human cancer including breast, kidney, colon, gastric, and others (Foster and Xu, 2003). Elevated PLD activity in human cancer cell lines suppresses apoptosis, promotes cell migration, and stimulates angiogenic signals – all important for progression to a malignant tumor (Hui et al., 2004; Zheng et al., 2006; Shi et al., 2007). My thesis has followed up on these studies and provides new insights into the role of PLD in nutrient signaling pathway.

1.1.1 PLD structure and localization

Phospholipase D (PLD) catalyzes the hydrolysis of the membrane phospholipid phosphatidylcholine (PC) to generate choline and phosphatidic acid (PA) (Fig. 1.1.1). There are two classic isoforms of PLD (PLD1 and PLD2) in mammals which occur as splice variants and they share about 50% identity of amino acid sequence. The newly identified mitochondrial PLD (mitoPLD) which localizes to the outer surface of mitochondria is poorly understood (Choi et al., 2006). Both PLD1 and PLD2 contain two highly conserved catalytic HKD domains which are necessary for enzymatic activity. These HKD domains contain the amino acid sequence HxKxxxxD, where the amino acids are Histidine (H), any amino acid (X), Lysine (K) and aspartic acid (D) (Exton, 2002). Deletion mutations in either one of the HKD domains in PLD1 or PLD2 exhibited no catalytic activity (Xie et al., 1998). Another motif, the plekstrin homology (PH) domain, plays a significant role in enzyme activity and protein localization since deletion or point mutations in this domain inhibited enzyme activity and disrupted normal localization (Sciorra et al., 2002; Sugars et al., 2002). In addition, there is one domain on both PLD isoforms which is responsible for phosphatidylinositol-4, 5-bisphosphate (PIP2) binding (Sciorra et al.,

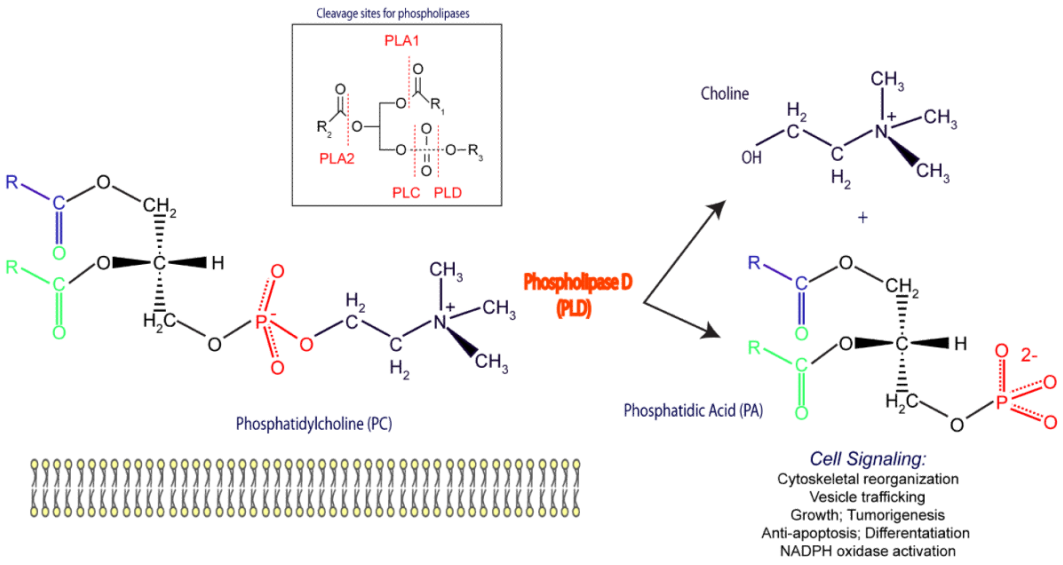

Figure 1.1.1 The reaction catalyzed by Phospholipase D. Phospholipase D (PLD) is responsible for catalyzing the hydrolysis of the membrane phospholipid phosphatidylcholine (PC) to generate choline and phosphatidic acid (PA) (Gomez-Cambronero, 2010).

1999). Another important motif is the phox homology (PX) domain which is responsible for protein-protein interactions and the association with phosphatidylinositol lipids which is proposed to be important for subcellular localization (Freyberg et al., 2001; Du et al., 2003). In addition, PLD1 contains a unique region known as the "loop" region which had been demonstrated to mediate the inhibition of PLD1 activity, this loop domain is absent in PLD2 (Sung et al., 1999) (Fig. 1.1.2).

It has been a controversial topic for many years as to the intracellular localization of different PLD isoforms since several groups characterized their distribution through overexpression of tagged proteins due to their low abundance. It was reported that PLD1 is tightly bound to endomembranes, whereas PLD2 is associated with the plasma membrane (Hammond et al., 1997; Colley et al., 1997). GFP or HA tagged PLD1 was observed in several organelles, such as lysosome and secretory granules, but little was detected in the Golgi apparatus (Brown et al., 1998; Toda et al., 1999). Hughes and Parker reported that PLD1

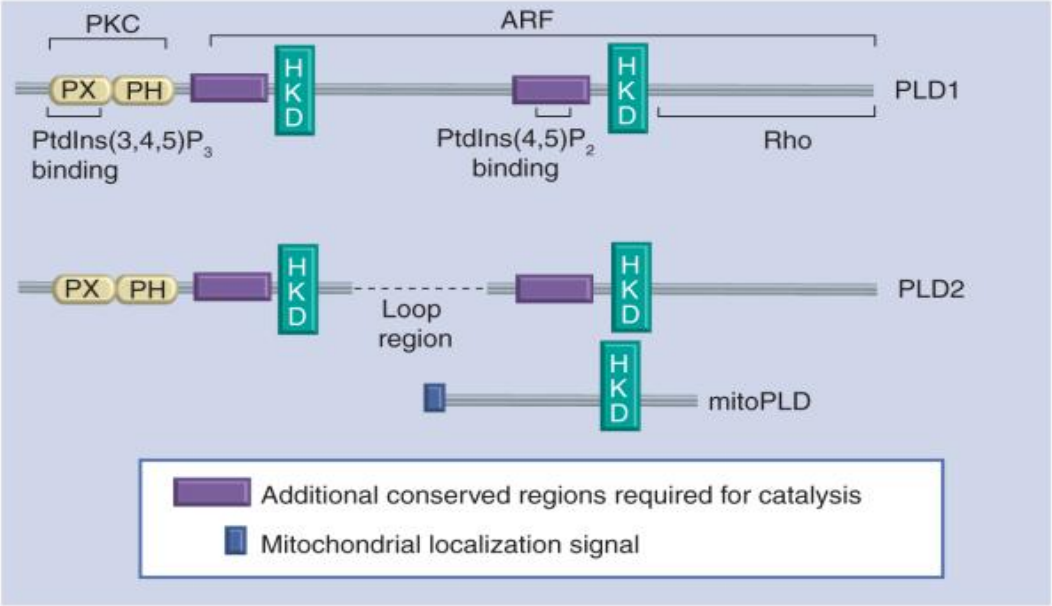

Figure 1.1.2 Basic structure of mammalian PLD1 and PLD2. HKD domains are required for catalytic activity. The PX and PH domains are lipid-binding domains which are important for PLD localization and activities. The loop region, which is absent in PLD2, negatively regulates PLD1 activity. The sites where PLD1 interacts with its regulators are also highlighted (Su et al., 2009).

localizes in endosomal compartments which is defined by the C-terminus (Hughes and Parker, 2001). Freyberg and colleagues showed that endogenous PLD1 was enriched significantly in the Golgi apparatus and surprisely was also present in cell nuclei (Freyberg et al., 2005). Since PLD1 and PLD2 are downstream effectors for many diverse cellular signaling events, it is very possible that they display dynamic subcellular localization which alters dramatically in response to different stimuli and in different cells.

1.1.2 PLD regulation

There are two PLD isoforms (PLD1 and PLD2) in mammals which differ in basal activity and regulatory interactions. Most attention has focused on PLD1 since it has a low basal activity that increases significantly in response to various stimuli. On the other hand, PLD2 has a high basal activity and its role in signalling is still elusive (Table 1.1.1). PLD activity is regulated by

many factors, including growth factors, small GTPases – ARF and Rho subfamily GTPases, protein kinase C (PKC), phosphatidylinositol-4, 5-bisphosphate (PIP2) and some other post-translational modifications. Many of these factors regulate PLD activity in either synergistic or antagonistic ways which make this regulatory network even more complicated.

Table 1.1.1 Properties of PLD1 and PLD2 (Foster and Xu, 2003).

	PLD1	**PLD2**
Molecular Weight	~120 kDa	~100 kDa
Substrate specificity	PC	PC
PIP_2-dependent	yes	yes
RalA- associated	yes	no
Basal activity	low	high
Subcellular localization	intracellular membranes	plasma membrane
Activators	Rho, ARF, PKC	ARF

Mammalian PLD proteins have been numerously reported to respond to growth factors. Many reports showed strong evidence that PLD activity can be stimulated by epidermal growth factor (EGF), platelet derived growth factor (PDGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and insulin in different model systems (Song et al., 1994; Yeo and Exton, 1995; Banno et al., 2003). Members of the Rho and ARF subfamily play critical roles in PLD activation. Rho family members (RhoA, Rac1, CDC42) interact directly with PLD and also regulate its activity (Brown et al., 1993; Cockcroft et al., 1994; Bae et al., 1998). All of the ARF family GTPases (ARF1-6) have been reported to activate PLD activity *in vitro* ((Massenburg et al., 1994; Hammond et al., 1995; Exton 2002). RalA GTPase has also been proposed to regulate PLD activity independent of its GTP loading state by collaborating with

ARF (Jiang et al., 1995). Protein kinase C (PKC) is another well-studied activator of PLD in response to mitogenic signaling (Yeo and Exton, 1995; Hornia et al., 1999). PLD and PKC also physically interact, resulting in strong activation of PLD1 activity *in vitro* although the kinase activity is not required for this activation (Pai et al., 1991; Eldar et al., 1993; Hu et al., 2003). Inhibition of PKC leads to the decrease of receptor-induced PLD activity, and PLD1 mutants unresponsive to PKC also respond poorly to activation of GPCR (Zhang et al., 1999). PIP2 has been shown to act as critical cofactor for PLD, and profoundly influences the activity, membrane localization and receptor activation of both PLD1 and PLD2 (Brown et al. 1993; Hodgkin et al. 2000). Taken together, through complicated regulation network mediated by the small GTPases, PKC, PIP2 and others, PLD functions as a all-round player in cellular signaling events to contribute to cell growth, cell survival, vesicle transport, cytoskeleton dynamics and cell adhesion.

1.1.3 PLD function

The main functions of PLD in the cell are in two aspects, phospholipid turnover and signal transduction events through PA and its metabolites. As mentioned before, PLD is a phospholipid-metabolizing enzyme that can convert phosphatidylcholine (PC) to phosphatidic acid (PA) and choline. PA is a well-defined lipid second messenger and can be converted to other biomolecules, such as, LPA and DAG (Jenkins et al., 2005). Collectively, PLD has been implicated in a broad variety of physiological cellular functions, such as intracellular vesicle trafficking, cytoskeletal dynamics, receptor endocytosis, exocytosis, cell proliferation, cell migration and survival signals (Exton, 2002; Foster and Xu, 2003; Foster, 2007) (Fig. 1.1.3).

Cell proliferation

PLD responds to many diverse mitogenic signals - epidermal growth factor (EGF),

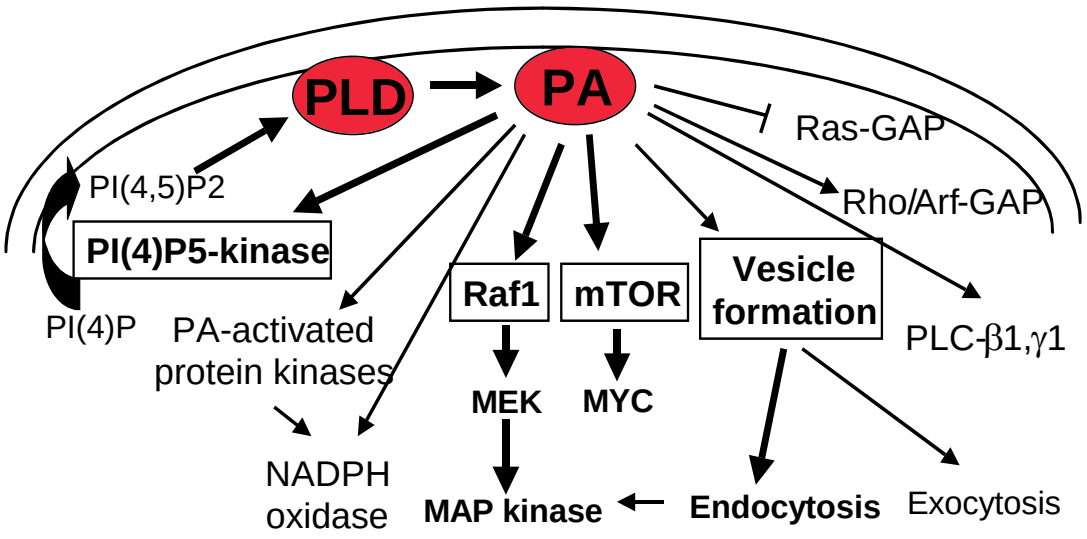

Figure 1.1.3 Functions of phosphatidic acid generated by PLD. Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA) which is believed to be important lipid second messenger. PA is actively involved in many diverse cellular activities (Foster, 2009).

platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), insulin, lysophosphatidic acid (LPA), and spingosine 1-phosphate, all of which can directly interact with either G-protein coupled receptors (GPCR) or receptor-tyrosine kinases (RTK). PLD activation stimulated by these mitogenic signals is actively involved in the regulation of cell proliferation, cell survival and the inhibition of apoptosis (Foster et al., 2003; Lee et al., 2009; Su et al., 2009). PA, the metabolite of PLD activity, was also found to bind to mTOR-FRB domain and regulates its cell growth signaling activity (Fang et al., 2001). PA can interact with SOS and translocate it to the plasma membrane to mediate EGF signaling (Zhao et al., 2007). Recent study showed that PLD1 acts in concert with the Wnt/β-catenin/TCF–dependent pathway to promote anchorage-independent growth. In NIH3T3 cells, it was found that increased anchorage-independent colony growth is significantly suppressed by PLD inhibitor and PLD

depletion with siRNA (Kang et al., 2010).

Vesicle trafficking

Many labs had reported that PLD is actively involved in vesicle formation and trafficking, such as endocytosis and exocytosis. Some reports have suggested that PA generated by PLD can contribute to exocytosis including mast cell degranulation, insulin release and EGF secretion (Hitomi et al., 2004; Hughes et al., 2004). Moreover, PLD has also been shown to be required for exocytotic processes in several different cell lines, such as adipocytes, neuroendocrine cells, mast and pancreatic B-cells. PA can recruit phosphatidylinositol 4-phosphate 5-kinase (PIP5K) which regulates vesicle fusion and the inner membrane curvature (Jenkins and Frohman, 2005). Furthermore, endocytic processes including receptor mediated endocytosis and phagocytosis are also dependent on PA generated by PLD (Hughes et al., 2004; Lyer et al., 2004; Antonescu et al., 2010). It was suggested that PLD protein, not PA can enhance the GTPase activity of dynamin which plays an important role for EGFR endocytosis (Lee et al., 2006).

Cytoskeleton rearrangement

PA has been demonstrated to be a key regulator of cytoskeleton rearrangements to promote cell adhesion, spreading, and migration. PLD is regulated by kinases (PKC and PIP5K) and small GTPases (Rho, Rac, cdc42, ARF, and RalA) that are actively involved in and actually required for cytoskeleton rearrangement (Rudge et al., 2009). Moreover, PLD product phosphatidic acid (PA) directly binds with Rac1 and acts as a membrane anchor for Rac1. Rac1 mutated in the region which is responsible for interaction with PA is incapable of translocating to the plasma membrane and inducing integrin-mediated cell spreading (Chae et al., 2008). Recently, PLD-generated PA was demonstrated to contribute to the increased local actin polymerization by recruiting the guanine nucleotide exchange factor of Rac1, DOCK2, to the

leading edge of polarizing cells (Nishikimi et al., 2009).

Cell differentiation

PLD appears to contribute to the differentiation processes in a variety of cell types. The differentiation of keratinocytes is correlated with sustained PA generation by PLD activation (Jung et al., 1999), and the expression level of PLD1 and PLD2 changes during granulocytic differentiation (Di Fulvio et al., 2005). PLD was also reported to have a significant role for neuronal cell differentiation (Kanaho et al., 2009). Moreover, Yoon et al. reported that during induction of differentiation in mouse C2C12 satellite cells, the expression of both PLD1 and PLD2 is elevated. C2C12 differentiation is remarkably inhibited by 1-butanol, an inhibitor of the PLD-catalyzed transphosphatidyla tion reaction, and also by the knockdown of PLD1 (Yoon et al., 2008).

1.1.4 PLD in cancer

Elevated PLD activity and expression have also been reported in various types of human cancer, including breast, colon, gastric and kidney (Huang and Frohman, 2007; Foster, 2009). For example, our lab showed that the expression and activity of PLD1 are upregulated in several breast cancer cell lines. Expression level of PLD2 is correlated directly with tumor size and survival of patients in colorectal carcinoma (Saito et al., 2007). These studies provide strong evidence that elevated PLD activity in cancer is functionally associated with tumor initiation and progression.

PLD in cell growth and survival

PLD activity was reported to suppress stress-induced apoptosis and provide a survival signal in breast cancer cell lines. Our lab reported that inhibiting PLD activity in MDA-MB-231 cells, a breast cancer cell line with highly elevated PLD activity, resulted in

apoptosis (Zheng et al., 2006). Moreover, inhibition of PLD activity in the T24 and Calu-1 cells resulted in apoptotic cell death in the absence of serum, therefore indicating that the elevated PLD activity plays a key role for cell survival in these two cancer cell lines (Shi et al., 2007). PLD1, but not PLD2, was shown to be required for Rheb activation of the mTOR pathway which is a key regulator for cell growth and proliferation (Sun et al., 2008). Taken together, all these studies indicate that PLD contributes to both cell proliferation and survival.

PLD and cell cycle regulation

Venable and colleagues proposed that the activation of PLD is critical for mitogenesis. The inhibition of PLD by elevated ceramide is closely linked with cellular senescence (Venable et al., 1999). Joseph and colleagues reported that PLD signaling provides a survival signal by overcoming cell cycle arrest induced by high intensity Raf signaling (Joseph et al., 2002). In addition, PLD also stimulates cell cycle progression by suppressing the expression of cyclin-dependent kinase inhibitor p21 (Kwun et al., 2003). TGF-beta signals that block cell cycle progression in G1 are suppressed in MDA-MB-231 cells at least partially by elevated PLD activity (Gadir et al., 2007). This finding indicates that the survival signal generated by PLD is also involved in the regulation of cell cycle progression mediated by mTOR.

PLD and metastasis

Having the ability to metastasize to other sites is the most critical step in progression to malignancy and is believed to be the main cause of mortality in cancer patients. Our lab showed that in breast cancer cell lines the ability of migration and invasion is directly related with the level of PLD activity. MDA-MB-231 human breast cancer cells, with high levels of PLD activity can migrate and invade the Matrigel in culture, whereas MCF-7 breast cancer cells with relatively low PLD activity can not (Zheng et al., 2006). In EL4 lymphoma cells, active PLD2

stimulates FAK phosphorylation, akt activation and cell invasion, whereas catalytically inactive PLD2 appears to have inhibitory effects on adhesion, migration and invasion (Knoepp et al., 2008). Park et al. demonstrated that the increased expression and activity of PLD in the glioma stimulate the secretion and expression of matrix metalloproteinase (MMP)-2, thus contributing to the invasiveness of glioma cells (Park et al., 2009). The connection between PLD and cytoskeleton organization, protease secretion, cell migration and invasion strongly suggests that PLD plays an important role in metastasis of cancer cells.

1.2 MAMMALIAN TARGET OF RAPAMYCIN (MTOR)

As mentioned before, PLD is actively involved in a broad variety of cellular activities including cell growth and proliferation, survival, angiogenesis and many others. These important cellular functions modulated by PLD are mediated, at least in part, by mTOR (the mammalian target of rapamycin), which is well known as central regulator for cell growth and proliferation and deregulated in many human cancers (Fang et al., 2001; .Foster, 2007; Foster, 2009). Since mTOR has been widely implicated as key driver for cell growth in human cancer cells (Sawyers, 2003; Guertin and Sabatini, 2007), the ability of PA generated by PLD to positively regulate mTOR implicates that mTOR is very likely an important component of PLD signaling in cell metabolism.

1.2.1 mTOR structure and complexes

TOR (target of rapamycin) was first discovered as the binding protein of the immosuppressant rapamycin that is produced by a soil bacterium found on Easter Island. TOR is a serine/threonine kinase highly conserved from yeast to mammals. Human, mouse and rat mTOR (mammalian target of rapamycin) proteins share above 90% identity at the amino acid level (Abraham, 2002; Jacinto and Hall, 2003). mTOR is considered to belong to the

PI3K-related protein kinase family since its C-terminus is highly homologous to the catalytic domain of PI3K (Gingras and Sonenberg, 2001; Manning et al., 2002). The kinase domain is located at the C-terminus and is responsible for the catalytic activity of mTOR. The HEAT repeats located at the N-terminus which mediate protein–protein interactions (Perry and Kleckner, 2003); and the FAT domain in the middle is involved in mediating mTOR interaction with other proteins and modulating the catalytic kinase activity of mTOR. The FRB domain is where the FKBP12 and rapamycin complex binds and inhibits the mTOR activity; and FATC domain also sits at C-terminus (Loewith et al., 2002; Yang and Guan, 2007) (Fig. 1.2.1).

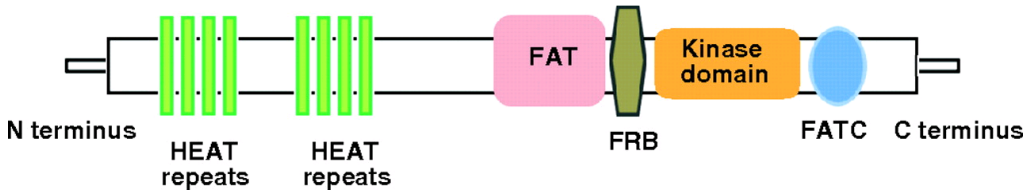

Figure 1.2.1 Schematic structure of mTOR. mTOR contains a C-terminal kinase domain which is similar to the kinase domain of PI3K family. N-terminal HEAT repeats are believed to mediate protein-protein interaction. FAT and FATC domains are responsible for modulating kinase activities. FRB domain is where FKBP12-rapamycin binds (Russell et al., 2011).

mTOR is the catalytic subunit of two biochemically and functionally distinct complexes called mTORC1 and mTORC2 (Corradetti and Guan, 2006; Wullschleger et al., 2006) (Fig. 1.2.2). Two unique components, regulatory-associated protein of mTOR (RAPTOR) and rapamycin-insensitive companion of mTOR (RICTOR), define mTORC1 and mTORC2, respectively (Hara et al., 2002; Kim et al., 2002; Sarbassov et al., 2004). Raptor and Rictor function as scaffolding proteins for the complexes assembly and for the recruitment of substrates and regulators. The common components which mTORC1 and mTORC2 share are mLST8/GβL and DEPTOR, which function as positive and negative regulators (Harris and Lawrence, 2003; Peterson et al., 2009). In addition, mTORC1 associates with PRAS40 (proline-rich Akt/protein

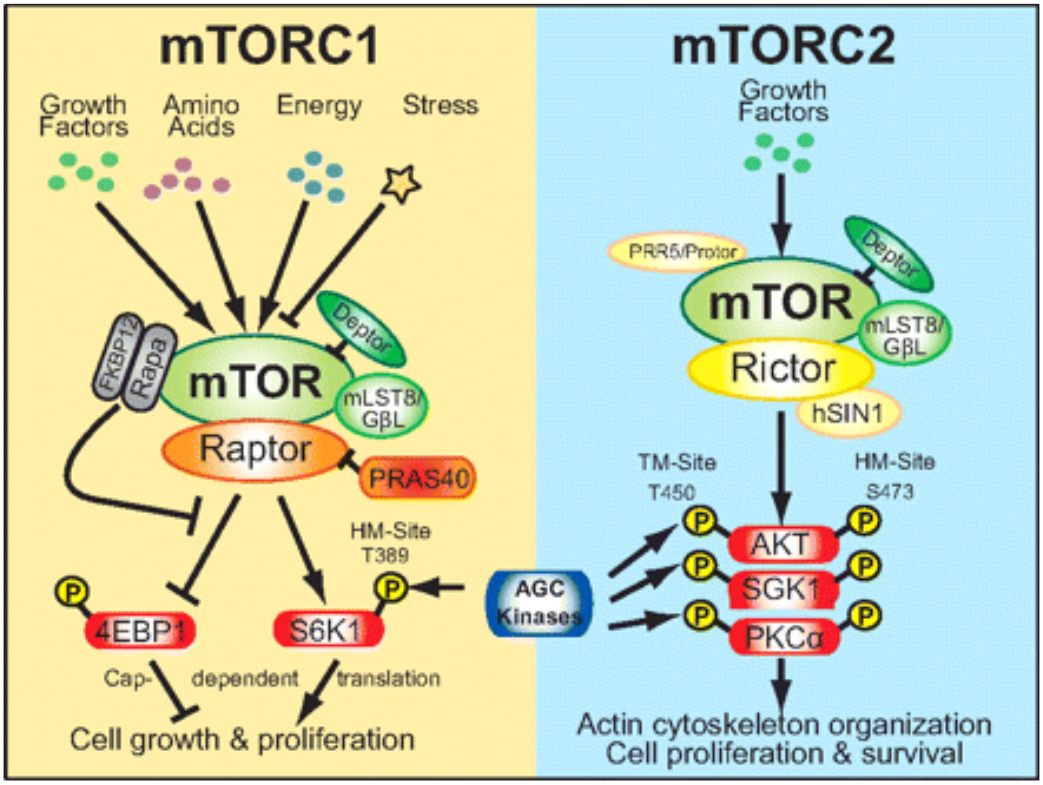

Figure 1.2.2 mTORC1 and mTORC2. These two complexes have distinct rapamycin sensitivitity, complex components, substrates which define their different cellular functions (Foster and Fingar, 2010).

kinase B (PKB) substrate 40 kDa), FK506-binding protein 38 (FKBP38) and Rag GTPases (Bai et al., 2007; Sancak et al., 2007; Wang et al., 2007; Ma et al., 2008; Sancak et al., 2008), whereas mTORC2 contains PROTOR and mSIN1 which are likely to help complex assembly and target mTORC2 to membranes (Jacinto et al., 2006; Yang et al., 2006; Pearce et al., 2007).

1.2.2 mTOR regulation

mTORC1 acts as a signal mediator for several major regulatory inputs, such as growth factors, nutrients, energy and stress (Sarbassov et al., 2005; Sengupta et al., 2010; Zoncu et al., 2011). These inputs work collaboratively or antagonistically to fine-tune mTORC1 activity (Fig. 1.2.3). Surprisingly the mechanism by which mTORC2 is regulated remains elusive. Due to its role in regulating AKT, SGK and PKC (Guertin et al., 2006; García-Martínez and Alessi D, 2008;

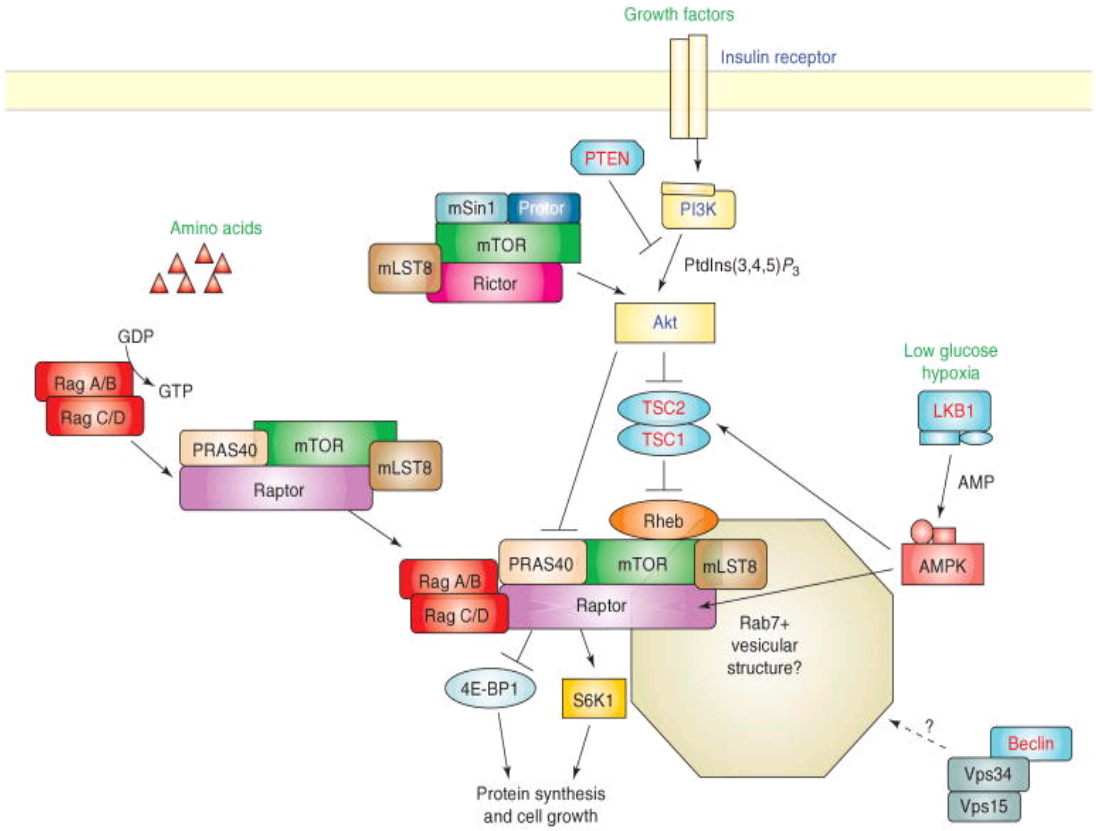

Figure 1.2.3 mTORC1 activation in response to nutrients and growth factors. Growth factors activate mTORC1 through PI3K/TSC/Rheb pathway. TSC2 and TSC1 form a complex that functions as a GAP for the Rheb GTPase. Akt and other kinases phosphorylate several residues in the TSC2 tumor suppressor, leading to its inactivation. Therefore elevated GTP-bound active Rheb will interact with mTORC1, thereby stimulating its kinase activity. Meanwhile, new studies identified the Rag GTPases as critical mediators of amino acid signaling to the mTORC1 complex. Upon amino acid stimulation the Rag-bound mTORC1 complex translocated to Rab7-positive perinuclear vesicular structures where Rheb also resides. The recruitment of mTORC1 driven by Rag GTPases allows Rheb to bind mTORC1 and stimulate its kinase activity. Vps34, sole member of class III PI3K, was reported to function as another important signal mediator which links amino acids to mTORC1 activation (Shaw, 2008).

Ikenoue et al., 2008), it is generally believed that growth factors modulate mTORC2 activity, directly or indirectly.

In response to increased availability of nutrients, especially leucine, mTOR signaling is

activated indicated by the elevated level of phosphorylated S6 kinase (S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), two substrates of mTORC1 (Kim et al., 2002). In the absence of nutrients (amino acids or glucose), mTORC1 activity is dramatically inhibited, indicated by quick dephosphorylation of S6K and 4E-BP1 with the deprivation of nutrients. Although the amino acid sensor is still unknown, candidate mediators acting downstream of amino acids have been suggested, including Rag family of small GTPases, mitogen-activated protein kinase kinase kinase kinase 3 (MAP4K3), RalA, and Class III PI3K catalytic subunit (also known as Vps34). Maehama and colleagues demonstrated that RalA and its activator RalGDS participate in nutrient sensing and are required for activation of mTORC1 in response to extracellular nutrients. The depletion of either RalA or RalGDS with siRNA abolished amino acid- and glucose-induced mTORC1 activation, as indicated by decreased phosphorylation level of S6K and 4E-BP1 (Maehama et al., 2008). The class III PI3 kinase, Vps34, was implicated in mediating the signals from amino acids to mTORC1, however *in vivo* study in flies with Vps34 mutants did not support this role (Nobukuni et al., 2005; Gulati et al., 2008; Juhasz et al., 2008). The STE20 kinase family member MAP4K3 was also highlighted as a regulator of S6K and 4E-BP1 activity in response to amino acids, but not to growth factors or insulin (Bryk et al., 2010; Yan et al., 2010; Esnik-Docampo and De Celis, 2011). The localization of mTOR was shown to be critical for its function. The Sabatini group demonstrated that Rag GTPases mediate relocating mTOR to Rab7-positive perinuclear vesicular structures where Rheb resides upon readdition of amino acids to starved cells, therefore implicating the important role of Rag GTPases for mediating amino acid signaling (Kim et al., 2008; Sancak et al., 2008; Shaw, 2008; Kim and Guan, 2009).

Growth factors stimulate the mTORC1 activation mainly through Ras-ERK and

PI3K-AKT pathways. Akt activates mTORC1 by phosphorylating and therefore inhibiting TSC2 (Tuberous Sclerosis Complex) activity, which binds with TSC1 to form the tuberous sclerosis complex (TSC). TSC negatively regulates mTORC1 activity by functioning as GAP (GTPase-activating protein) protein to inhibit a small GTPase called Rheb which directly associates and activates mTORC1 (Long et al., 2005). In addition to growth factors, TSC mediates signals from oxygen- and energy-sensing pathways as well to modulate mTORC1 activity.

The mTORC1 pathway also senses energy level by a mechanism that is mediated by the AMP-activated protein kinase (AMPK). Both AMP and ATP are allosteric regulators of AMPK, when the AMP levels rise and ATP levels drop, activated AMPK phosphorylates TSC2 at Thr1227 and Ser1345, which enhances its GTPase activity toward Rheb, thereby inactivating Rheb GTPase and inhibiting mTORC1 signaling pathway (Corradetti et al., 2004; Inoki et al., 2006). Moreover, AMPK has been shown to directly phosphorylate mTOR binding partner raptor at both Ser722 and Ser792, facilitating the binding between raptor and 14-3-3 proteins, which finally leads to the inhibition of mTORC1 signaling (Gwinn et al., 2008). The link between AMPK and mTORC1 activity allows the cell to coordinate energy consuming processes with energy level – When energy level is low, AMPK is activated to suppress the mTORC1 activity to further limit energy-consuming processes such as protein synthesis controlled by mTORC1.

1.2.3 mTOR function

mTOR functions by forming two complexes mTORC1 and mTORC2 with different subunit composition which determine their substrate specificity and cellular function. The mTORC1 substrates S6K1 and 4E-BP1 are involved in mRNA translation initiation and

progression, therefore regulating the rate of protein synthesis. When phosphorylated by mTORC1, S6K1 promotes mRNA translation by phosphorylating or binding multiple proteins, such as the ribosomal S6 protein and eIF-4B, which work together to regulate translation initiation and elongation process (Sabatini, 2006). Unphosphorylated 4E-BP1 suppresses cap-dependent mRNA translation by binding to the eukaryotic translation initiation factor 4E (eIF4E) protein and preventing it from interacting with the translation initiation factor; however, when phosphorylated by mTORC1, it releases eIF4E which participates in recruiting eIF4F complex assembly and promoting cap-dependent protein synthesis (Ma and Blenis, 2009).

TORC2 was first identified in S. cerevisiae to mediate actin cytoskeleton organization and cell polarization. This role has been further confirmed in D. discoideum and mammalian cells (Jacinto et al., 2004; Sarbassov et al., 2004). Recent findings have discovered novel roles for mTORC2 in the phosphorylation and activation of AGC kinase family members including Akt, serum- and glucocorticoid-regulated kinase (SGK), and protein kinase C (PKC) (Guertin et al., 2006; Ikenoue et al., 2008; García-Martínez and Alessi, 2008). Through these downstream substrates mTORC2 regulates cell growth, cell survival, cell cycle progression and metabolism.

1.2.4 mTOR in cancer

The signaling components that regulate mTOR activity are frequently mutated in human cancers. For example, PTEN loss in human cancers occurs with high frequency only next to that of the well-known tumor suppressor p53 (Yuan and Cantley, 2008). In addition, the catalytic subunit of PI3K-p110α is mutated in a variety of human cancers (Vivanco and Sawyers, 2002). Ras, which binds and activates the catalytic subunit of PI3K, is overactivated in about 20–30% human tumors (Downward, 2003). Moreover, tumor suppressor TSC2 and its binding partner TSC1 are mutated in an autosomal dominant disorder known as tuberous sclerosis complex

(TSC). TSC patients are predisposed to widespread benign tumours termed hamartomas in kidney, lung, brain and skin (Jones et al. 1999). These findings provide compelling evidence that the deregulation of mTOR pathway plays a critical role in tumorigenesis.

The mechanisms by which mTOR contributes to cancer development is through its effect on cell proliferation, cell cycle progression in combination with metastasis (Guba et al., 2002; Sabatini, 2006; Efeyan and Sabatini, 2010). There is strong evidence that mTOR is required for cell cycle progression, and inhibition of mTOR activity by rapamycin leads to cell cycle arrest in the G1 phase of the cell cycle (Fingar and Blenis, 2004). Expression of a rapamycin-resistant mutant of mTOR relieves the effect of rapamycin on cell cycle progression, moreover Blenis and colleagues reported that the effect of rapamycin on cell cycle progression is exerted through the inhibition of the downstream effectors of mTOR, S6K and 4E-BP1 (Fingar et al. 2004). The mTORC1 pathway is also important for regulating cyclin D1 transcription, translation and stability in many types of cancer cell lines (Gao et al., 2004; Law et al., 2006). Guba et al. reported the inhibitor of mTOR -rapamycin suppressed metastatic tumor growth and angiogenesis in *in vivo* mouse models. It was reported that mTORC1 regulates the translation and activity of HIF1α which further facilitates expression of vascular endothelial growth factor (VEGF), indicating that the antiangiogenic properties of rapamycin could result from its ability to interrupt VEGF expression (Bernardi et al., 2006). Since the early 1980s that rapamycin was found with anti-tumor properties, it has taken many years for our good understanding the complicated relationship between mTOR and cancer. These exciting findings allow us to have a better view of the role that mTOR plays in cancer and opening the door to new therapeutic strategies.

1.2.5 mTOR and PLD

For the last ten years a significant finding for PLD was the discovery of PA requirement for mTOR (Fang et al., 2001). Later on the accumulation of data from several laboratories has made it obvious that PA is an important regulator of mTOR (Chen et al., 2003; Hornberger et al., 2006; Toschi et al., 2009).

Fang et al. first reported that PA can physically associates with the FKBP12-rapamycin-binding (FRB) domain of mTOR. This lipid-protein interaction is highly specific for PA, and could be abolished by the FKBP12-rapamycin complex. Furthermore it was showed by mutation analysis that Arg2109 in FRB is critical for this interaction between FRB domain of mTOR and PA. This group further demonstrated the relationship between PLD and mTOR by showing that the elimination of endogeneous PLD1 by siRNA led to dramatic inhibition of serum-stimulated S6K1 and 4E-BP1 phosphorylation in both HEK293 and COS-7 cells. This result is also confirmed by a catalytically inactive dominant-negative PLD1 plasmid (Fang et al., 2003). Consistent with data from the Chen group, our lab showed that elevated PLD activity in human breast cancer cells conferred rapamycin resistance which also indicates that PA and rapamycin compete for binding with FRB domain of mTOR (Chen et al., 2003). Another convincing evidence from the solution structure of PA-FRB complex supports the mutually exclusive model of FRB binding for PA and FKBP12-rapamycin, and the crucial role of Arg2109 for this binding (Veverka et al., 2008).

The Rheb GTPase and TSC act as positive and negative regulators of mTORC1 signaling respectively. Chen's lab recently uncovered a direct interaction between Rheb and PLD. They showed convincing data that Rheb binds and activates PLD1 *in vitro* in a GTP-dependent manner. PLD activation by mitogen stimulation is attentuated by Rheb knockdown or TSC2 overexpression, suggesting that TSC-Rheb is an indispensable upstream regulator of PLD (Sun

et al., 2008). All together, the data linking PLD with mTOR activation represents a novel and potentially very important mechanism for activating this critical signal integrator by which multiple inputs including cellular energy levels, growth factors, and hypoxia converge to fine tune many cellular activities.

1.3 Ras superfamily

PLD activity is tightly regulated by a wide range of cellular factors and many of them belong to Ras superfamily small GTPases (Feig, 2003; Foster and Xu, 2003). For example, Rho and ARF subfamily play key roles in PLD activation. Ral GTPase contributes to the activation of PLD by recruiting PLD close to its well defined activator ARF6 (Luo et al., 2007). Rheb directly interacts with PLD, resulting in strong activation of PLD1 activity *in vitro* (Sun et al., 2008). Overall, the small GTPases are closely related with PLD and appear crucial for the localization and activity of PLD. Moreover, several members of Ras superfamily have been implicated in regulating mTOR activity. Rheb is a well-defined upstream regulator of mTOR. Rag GTPases is required for the translocation of mTOR to endomembranes to be further activated upon the stimulation of amino acids (Sancak et al., 2008). Recently Saci and colleagues reported that Rac1, a member of the Rho family of GTPases, is a key regulator of both mTORC1 and mTORC2 in response to growth-factor stimulation (Saci et al., 2011). It was showed that TORC1 activity is regulated by members of the Rab and ARF family GTPases in Drosophila. In mamalian cells, overactivation of Rab5 and ARF1 can significantly inhibit amino acid induced mTORC1 activation. All these finding demonstrate the important role of Ras superfamily in modulating mTOR activity. Therefore it will be of particular interest as to whether they also participte in nutrient signaling mediated by PLD.

The Ras superfamily of small GTPases are divided into five subfamilies-Ras, Rho, ARF (ADP ribosylation factor), Ran and Rab GTPases based on their sequence and functional

similarities (Colicelli, 2004) (Fig. 1.3.1). These GTPases function as key regulators in signaling pathways that contribute to actin cytoskeleton organization, cell polarity and movement, vesicular transport, cell cycle regulation and cell survival (Kinbara et al., 2003; Ridley et al., 2003; Colicelli 2004; Mitin et al., 2005).

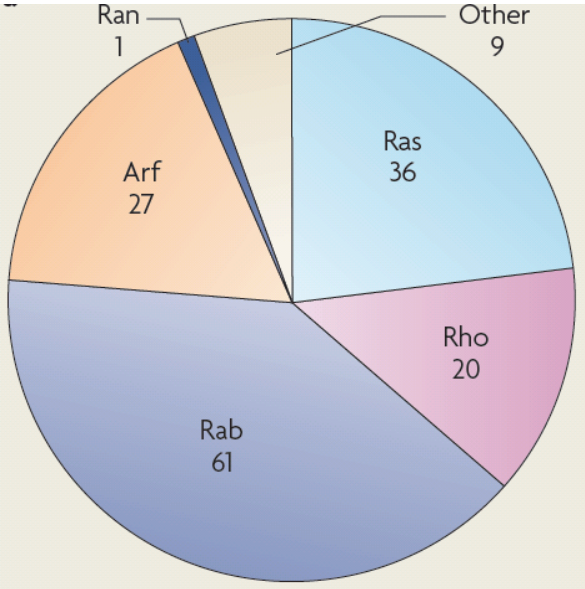

Figure 1.3.1 Diagram of Ras superfamily GTPases. The Ras superfamily of small GTPases constitutes by more than 150 proteins which are divided into five major branches, including Ras, Rho, Rab, Ran and Arf GTPases (Vigil et al., 2010).

1.3.1 Regulation of small GTPase

Small GTPases are molecular switches that exist in a GTP-bound 'on' state or a GDP-bound 'off' state. The GTP–GDP cycle is executed by regulators of small GTPases, such as GEFs (guanine nucleotide exchange factors) and GAPs (GTPase-activating proteins) (Fig. 1.3.2). It was proposed that GEFs interact with the switch I and II regions of the GTPase and stabilize a conformation that disfavors GDP binding (Worthylake et al., 2000). However, the function of GEFs is not just to activate GTPases, it can also specify the downstream effectors of the activated GTPase by forming a scaffolding platform for them (Wiget et al., 2004). Small GTPases have their intrinsic activity to hydrolyse GTP. However, this activity is low and requires reinforcement by GAPs. GAPs stabilize the transition state of the GTPase reaction by inserting an ‘arginine finger’ into the GTPase, thereby significantly speeding up the process of GTP hydrolysis. Specifically for the Rho and Rab subfamilies, another level of regulation functions through GDIs (guanine nucleotide dissociation inhibitors) which interact with GDP-bound GTPases (DerMardirossian and Bokoch, 2005). These abundant and mostly

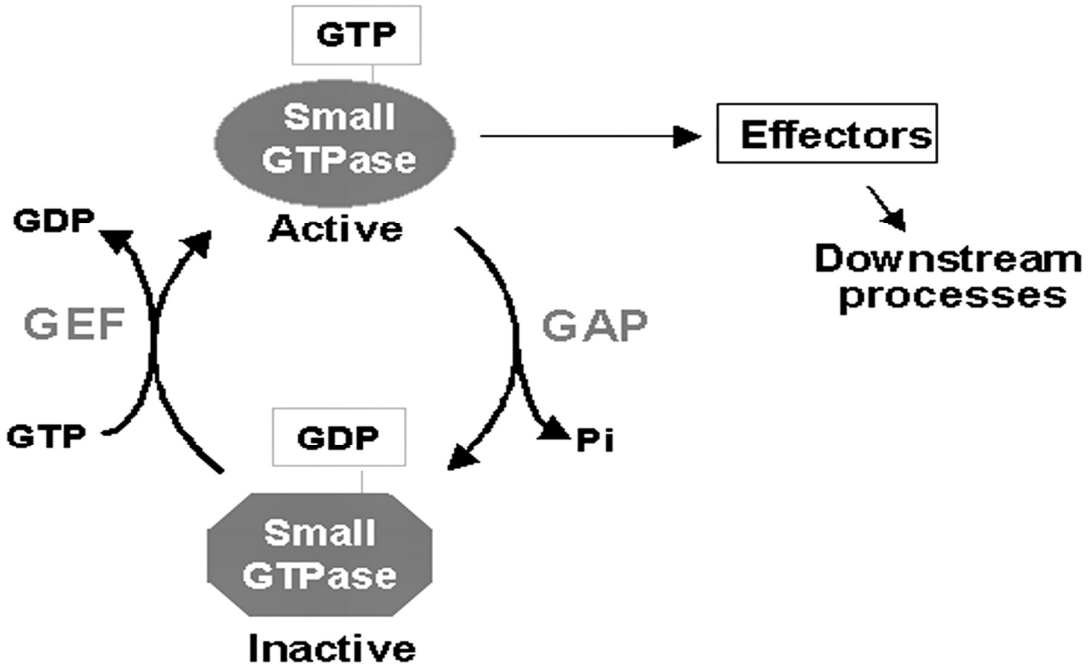

Figure 1.3.2 The GTP-GDP cycle of small GTPases. The Ras superfamily of small GTPases cycle between an active GTP-bound and an inactive GDP-bound state. GEFs activate these small GTPases, which further interact with specific effectors to mediate downstream pathways. GAPs enhance the intrinsic GTPase activity of these small GTPases, accelerating the inactivation of their regulatory activity (Nielsen et al., 2008).

cytosolic proteins prevent the dissociation of GDP by stabilizing GTPase and interfering their interaction with regulatory molecules and effector proteins (del Pozo et al., 2002; DerMardirossian et al., 2004).

1.3.2 Function of small GTPases

Among these five subfamilies, Rho GTPases was reported to actively participate in signal transduction, cytoskeleton organization, cell shape and polarity, cell–cell and cell–matrix interactions. Both Rab GTPases and ARF GTPases play a significant role in vesicular trafficking, regulating endocytosis and exocytosis pathways. The Ran GTPase participates in nuclear–cytoplasm transport and mitotic spindle organization. Ras GTPases are well known for their important role in cancer (Feig, 2003; Colicelli 2004). Two important subfamily- Ras and

ARF GTPases directly related with my project are discussed here in detail.

Ral GTPases

The Ral guanosine nucleotide-binding proteins, RalA and RalB, were discovered more than 20 years ago when Dr. Chardin isolated their cognate genes by using degenerate probes that contained highly conserved Ras sequences (Chardin and Tavitian, 1986). RalA and RalB which share the 80% identity to each other at the amino acid level represent the pivotal branch of the Ras superfamily (Colicelli, 2004). Following their initial discovery, numerous studies have subsequently explored their functions in cell regulatory networks. Because Ral guanosine nucleotide exchange factors (Ral-GEFs) are direct effectors of Ras, the Ral signaling pathway has been traditionally considered a Ras-effector pathway (Bodemann and White, 2008). The Ral GTPases are actively involved in oncogenic transformation, cell proliferation, endocytosis and vesicle transport (Feig, 2003; Camonis and White, 2005; van Dam and Robinson, 2006).

ARF GTPases

ADP ribosylation factor (ARF) GTPases are another important branch of the Ras superfamily of small GTPases. The ARF proteins can be divided into three classes: Class I – ARF 1-3; Class II – ARF 4, 5; Class III – ARF6. Class I and II ARF GTPases are more likely associated with trans-Golgi network (TGN), whereas the Class III protein ARF6 localizes at the plasma membrane and participates in vesicle formation at the plasma membrane, vesicle recycling and remodeling of the actin cytoskeleton (Cavenagh et al., 1996; Donaldson and Honda, 2005; Honda et al., 2005). All ARF GTPases are modified by myristoylation at the N-terminus and this modification is required for both membrane association and biological activities (Donaldson and Jackson, 2011).

Among all the ARF GTPases, ARF1 is the most abundant and best-characterized. For example, ARF1 participates in vesicle formation and budding by recruiting its effectors,

including coatomer and clathrin adaptor complex (AP), to the Golgi. ARF6 localizes mainly at the plasma membrane and regulates the remodeling actin cytoskeleton, phospholipid metabolism and receptor mediated endocytosis (Donaldson, 2003). For example, at the plasma membrane ARF6 activates PIP5K and PLD, resulting in the generation of PIP2 and PA which are critical for sorting proteins within the membrane and the formation of clathrin-coated pits during endocytosis (D'Souza-Schorey and Chavrier, 2006). Bach and colleagues found that ARF6 regulates myoblast fusion through PLD activation and PIP2 production (Bach et al., 2010). ARF6 was also reported to associate with endosomal membranes derived from clathrin-independent endocytosis and mediates recycling of this membrane back to the plasma membrane (Grant and Donaldson, 2009).

1.3.3 Small GTPases in cancer

Since all these small GTPases play a fundamental role in normal development and physiology, they will lead to life-threatening diseases, such as cancer, once deregulated (Vigil et al., 2010). In addition, the critical regulators of small GTPases, such as GEFs and GAPs, have been found to contain mutations that are associated with tumor initiation, progression and metastasis (Jaffe and Hall, 2002; Malliri and Cllard, 2003).

Ral proteins were shown to play a significant role in tumorigenesis (Lim et al., 2005; Zipfel et al., 2010; Martin et al., 2011). Several studies had attracted the attention to identify Ral activation contribute to Ras induced oncogenic transformation in cell culture (Ward et al., 2001; Rangarajan et al., 2004). Chronic activation of RalA and RalB occurs in tumour-derived cell lines and tumour samples (Lim et al., 2005; Chien et al., 2006). Depletion of RalA severely impedes the anchorage-independent proliferation of cancer cells, whereas RalB seems to be required for the survival of a wide range of tumour-derived cell lines (Falsetti et al., 2007). For example, RalA was reported to be constantly overactivated in all MPNST cells compared to

nontransformed Schwann cells. Both silencing Ral with siRNA and suppression its activation with a dominant-negative mutant Ral S28N caused a significant inhibition in proliferation, invasiveness of MPNST cells (Bodempudi et al., 2009).

ARF1 and ARF6, the most studied isoforms of the ARF GTPase subfamily, are regulators of many important cancer cell properties, such as cell proliferation, invasion, and resistance to apoptosis, especially in breast cancer cell lines (Boulay et al., 2008; Muralidharan-Chari et al., 2009). Dr. Clanig's group reported that ARF1 has a pivatol role in controlling cell proliferation. When they inhibited ARF1 activity by using dominate-negative mutant, they found that breast cancer cells showed sustained cell-growth arrest. In addition, they demonstrated that the GDP-bound form of ARF1 directly interacted with pRB. The depletion of ARF1 or expression of ARF1T(31)N resulted in the stable association between pRB and E2F1, thereby suppressing the expression of E2F target genes, such as cyclin D1, Mcm6 and E2F1which are important regulators for cell-cycle progression (Boulay et al., 2011). Collective evidence indicates that ARF6 contributes to cancer progression through activation of cell motility and invasion. Overexpression of ARF6 was reported in highly invasive breast cancer cell lines (Hashimoto et al., 2004). GEP100, a GEF for ARF6, has also been implicated in breast cancer invasion (Morishige et al., 2008). RNAi knockdown of GEP100 to inhibit ARF6 activation results in reduced breast cancer cell invasion by *in vitro* Matrigel assay and reduced metastasis in a breast cancer mouse model. The same group also reported that ARF1 regulates breast cancer cell growth and invasion during cancer progression by the activation of the phosphatidylinositol 3-kinase pathway (Boulay et al., 2008). Taken together, convincing evidence implicates there is a strong correlation between cancer development and deregulation of small GTPases, therefore shedding light on targeting small GTPases as new cancer therapy strategies.

1.3.4 Small GTPases and PLD

Our lab first demonstrated that RalA is involved in V-src induced activation of PLD by directly binding with PLD1 through its N terminus, and that PLD is a downstream target of a Ras/Ral GTPase signaling pathway (Jiang et al., 1995). Moreover, PLD1 was reported to be recruited by RalA GTPase and further activated by ARF6 GTPase and this activation is dependent on the GTP-loading status of ARF6 (Fig. 1.3.3) (Luo et al., 1997; Luo et al., 1998; Xu et al., 2003). PLD is involved in receptor-mediated endocytosis in which Ral GTPase also participate (Shen et al., 2001). The interaction between Ral and PLD is also implicated in neurite branching (Lalli and Hall, 2005). Deletion of the PLD binding sites in RalA or RalB demonstrates their different role: RalA in branching involves both PLD and exocyst interactions, while RalB-induced branching involves only PLD. In addition, the effect of RalA and RalB for branching is blocked by PLD inhibitors, indicating the crucial role of the Ral–PLD interaction in mediating neurite branching. Recent study from the Grant group revealed the participation of RalA and PLD in the regulation of FcgR-mediated phagocytosis (Corrotte et al., 2010). They reported that PLD activity is activated when phagocytosis happens, and RalA, but not RalB can block this activation. Moreover, RalA colocalized with PLD1 and PLD2 at the phagocytic cup during phagosome formation and there is enhanced interaction of RalA with both PLD isoforms during phagocytic stimulation. In summary, many studies showed that RalA works with PLD synergistically to regulate important cellular functions, such as endocytosis, vehicle transport and membrane trafficking.

It was reported that all ARF GTPases can activate PLD activity. Additionally, ARF GTPases always work together with RalA to activate PLD activity (Luo et al., 1998; Xu et al., 2003). Accumulating evidence showed that ARF6 directly binds to and activates PLD, thus

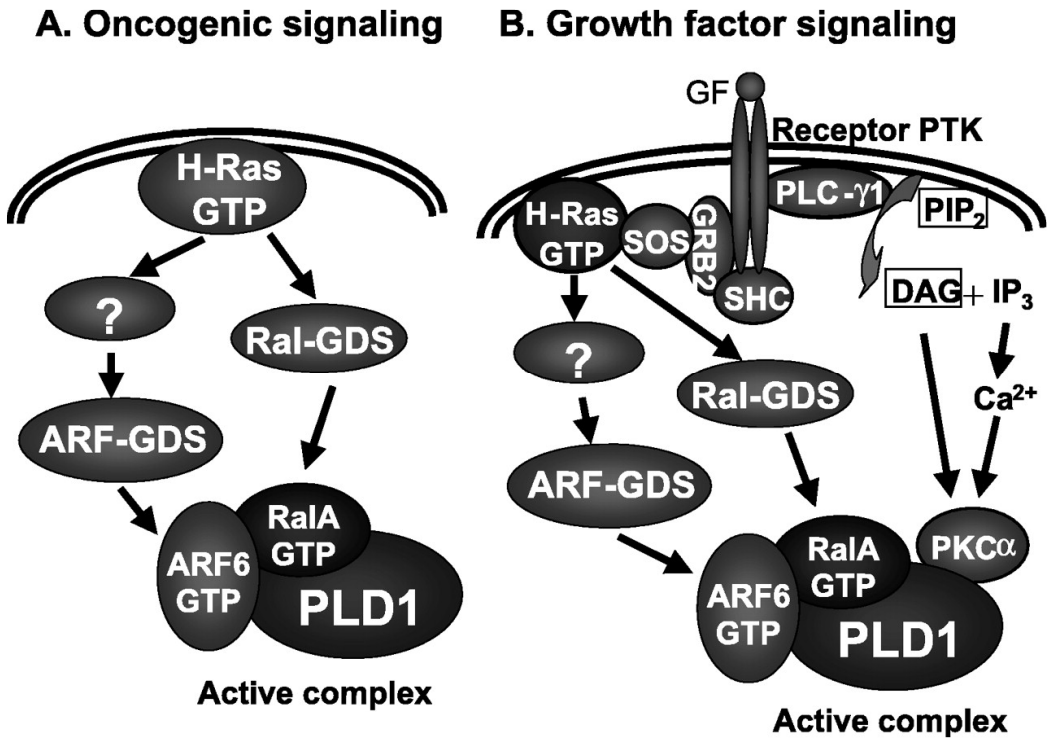

Figure 1.3.3 Activation of PLD1 through the synergistic action of RalA and ARF6. PLD is activated in response to oncogenic (A) or growth factors (B) stimulation. In both signaling pathways, the last step is that activated RalA recruits activated ARF6 into the RalA-PLD1 complex, thereby activating PLD1 activity (Foster and Xu, 2003).

regulating exocytosis, membrane ruffling, membrane recycling and other activities related with PLD (Dana et al., 2000; Powner et al., 2002; O'Luanaigh et al., 2002). Hiroyama and Exton reported that dominant positive ARF6 selectively activated PLD2 and dominant negative ARF6 selectively inhibited PLD2, indicating that PLD2 is selectively regulated by ARF6 in *vivo* (Hiroyama and Exton, 2005). Ma and colleagues investigated the role of PLD and ARF6 in insulin secretion stimulated by high glucose in the pancreatic Beta-cell line MIN6N8. Furtherfore they discovered that high glucose will induce the association between ARF6 and PLD, thus activating mTOR signaling pathway (Ma et al., 2010).

1.4 Class III phosphoinositol-3-kinase (PI3K)

PI3Ks are a family of related intracellular signal transducer enzymes which phosphorylate

the 3-hydroxyl group of the inositol ring on a wide variety of membrane associated phosphatidylinositols (PtdIns). PI3Ks signaling contributes to many important cellular activities, such as cell growth and survival, cell migration and intracellular vesicular transport, cell cycle progression (Engelman et al., 2006; Shaywitz et al., 2008). The PI3K family is divided into three different classes: Class I, Class II, and Class III. Each class of PI3-kinase responds to different signaling, has unique preferences for phosphoinositide substrates and produces specific lipid second messengers (Fig. 1.4.1) (Vanhaesebroeck et al., 2010). In addition, each class PI3-kinase functions by specific regulatory and catalytic subunits that associate into the heterodimers. Of particular interest is the class III phosphatidylinositol-3-kinase hVps34 which was recently reported as an important modulator for amino acid sensing mediated by mTOR (Byfield et al., 2005; Nobukuni et al., 2005; Backer, 2008).

1.4.1 Regulation of Vps34

The class III PI3K has only one catalytic member, vacuolar protein sorting 34 (Vps34; also known as hVps34 or PIK3C3 in human), which binds regulatory subunit Vps15 (also known as hVps15 or PIK3R4 in human) and uses PtdIns to generate PtdIns3P (PI3P). Vps34 can directly bind to the heat and protein kinase domains of Vps15. In addition Vps34 was proposed to be a substrate of Vps15, since Vps34 activity is abrogated in yeast expressing kinase-dead VPS15 mutant (Stack et al., 1993). Vps15 is myristoylated which will attach the Vps34-Vps15 complex to intracellular membranes (Herman et al., 1991). The recruitment and activity of the hVps34 on early and late endosomes are dependent on the upstream activation of the small GTPases Rab5 and Rab7, respectively (Murray et al., 2002; Stein et al., 2003).

It remians obscure whether Vps34 activity is regulated by extracellular stimuli, even though there is evidence that Vps34 activity could be modulated by nutrients such as amino acids

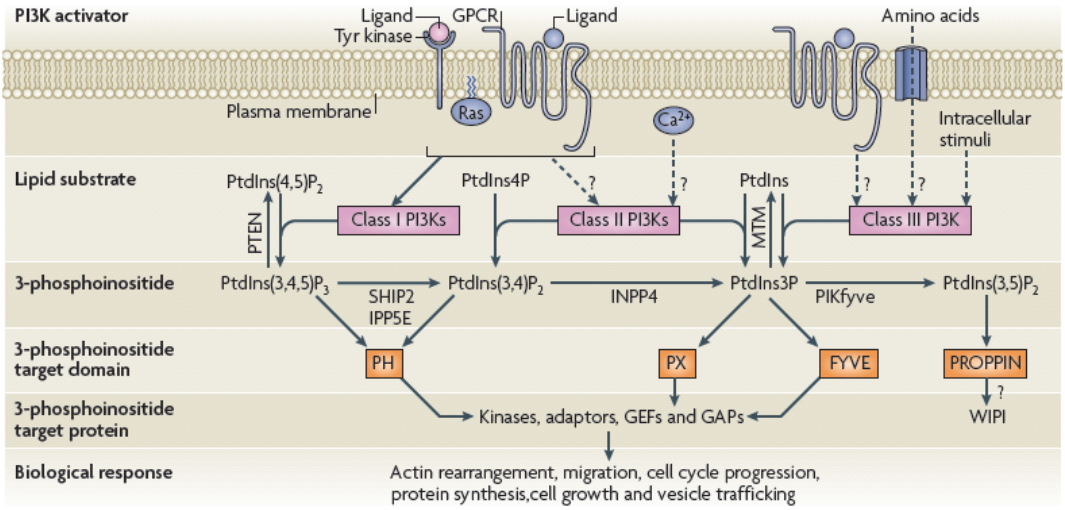

Figure 1.4.1 Characteristics of the PI3K family. The PI3K family is divided into three different classes: Class I, Class II, and Class III. Each class of PI3-kinase responds to different stimuli, has unique preferences for lipid substrates and generates specific lipid second messengers (Vanhaesebroeck et al., 2010).

and glucose and by GPCRs (Byfield et al., 2005; Nobukuni et al., 2005; Slessareva et al., 2006). For example, Windmiller and Backer reported that in mammals Vps34 is activated by Gα coupled receptor which signals downstream of the M1 muscarinic receptor (Windmiller and Backer, 2003). It was also proposed that Vps34 in yeast mediates the pheromone response through GPCR (Slessareva et al., 2006). Byfield and colleagues showed that hVps34 activity is affected by the availbility of amino acids and glucose, but not insulin, suggesting that hVps34 is a nutrient-regulated lipid kinase (Byfield et al., 2005). Moreover Vps34 can be negatively regulated by CDK (cyclin-dependent kinase) during cell cycle progression and development. Furuya and colleagues showed that the phosphorylation of Thr159 by CDK1 and CDK5 is a key regulatory event and possibly contributes to human diseases including neurodegeneration and cancers (Furuya et al., 2010).

1.4.2 Signaling of Vps34

Vps34 is a lipid kinase that specifically produces the lipid PI3P which is predominantly

enriched at early and late endosomes (Fig. 1.4.2) (Schu et al., 1993; Volinia et al., 1995). Vps34 coordinate the localization and function of many effector proteins, which bind PI3P by two specific lipid-binding domains: the PX domain and the FYVE domain (Stenmark et al., 2002; Lemmon, 2003).

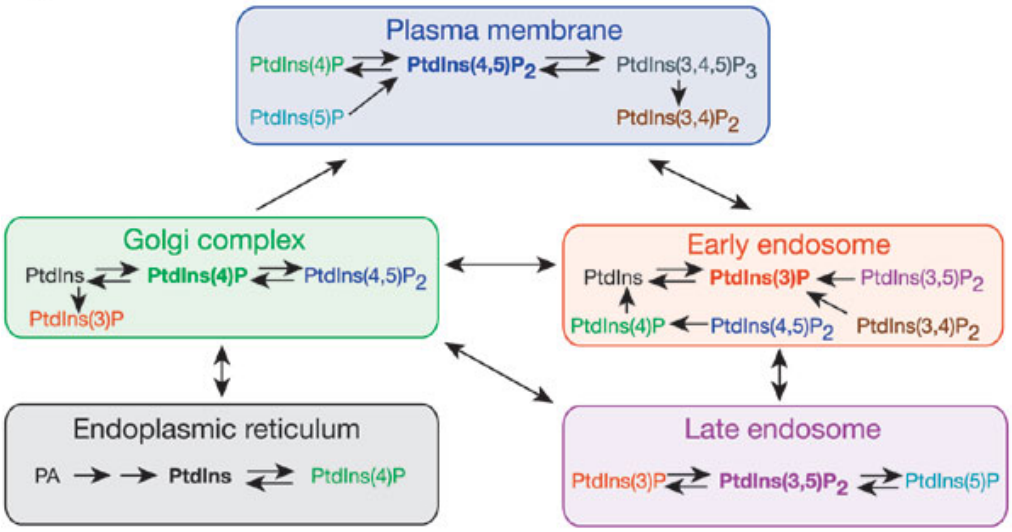

Figure 1.4.2 Subcellular localization of phosphoinositides. PtdIns(4,5)P_2 is mostly enriched at the plasma membrane. PtdIns(4)P is more likely found at the Golgi complex, less present at the plasma membrane. PtdIns(3)P is predominantly concentrated in early endosomes and small amount found at late compartments of the endosomal pathway. (Di Paolo and De Camilli, 2006).

FYVE zinc finger domain is named after the first four proteins found to contain this motif (Fab1, YotB, Vac1p, and EEA1) .The FYVE domain is about 70 amino acids which binds to the PI3P with high specificity and affinity and targets many FYVE domain-containing proteins to PI3P-enriched endocytic membranes (Gaullier et al., 1998; Lawe et al., 2000; Corvera, 2000). Therefore FYVE containing proteins actively participate in a broad variety of cellular functions including endocytosis, exocytosis, membrane trafficking and phosphoinositide metabolism (Chen et al., 2007; Hayakawa et al., 2007).

PX domain is named for the Phox homology domain of the $p47^{phox}$ and $p40^{phox}$ subunits of

the phagocyte NADPH oxidase in which it is first discovered. The PX domain is also a phospholipid-binding domain consisting of about 120 amino acids which interacts predominantly with PI3P and other lipids (Ago et al., 2001; Hiroaki et al., 2001; Wishart et al., 2001) (Fig. 1.4.3). The PX domains are found in more than 250 proteins, including the $p40^{phox}$ and $p47^{phox}$ components of the NADPH oxidase complex, sorting nexins, PLD and CISK (Xu et al., 2001; Stahelin et al., 2004; Lee et al., 2006; Koharudin et al., 2009). PX domains are highly conserved in structure although there is relatively little sequence similarity. Many proteins with PX domain have been reported to localize to PI3P-rich endosomal and vacuolar structures and implicated in a wide range of cellular functions such as signal transduction, vesicular trafficking, protein sorting and lipid modification (Ellson et al., 2002; Seet and Hong, 2006; Vanhaesebroeck et al., 2010).

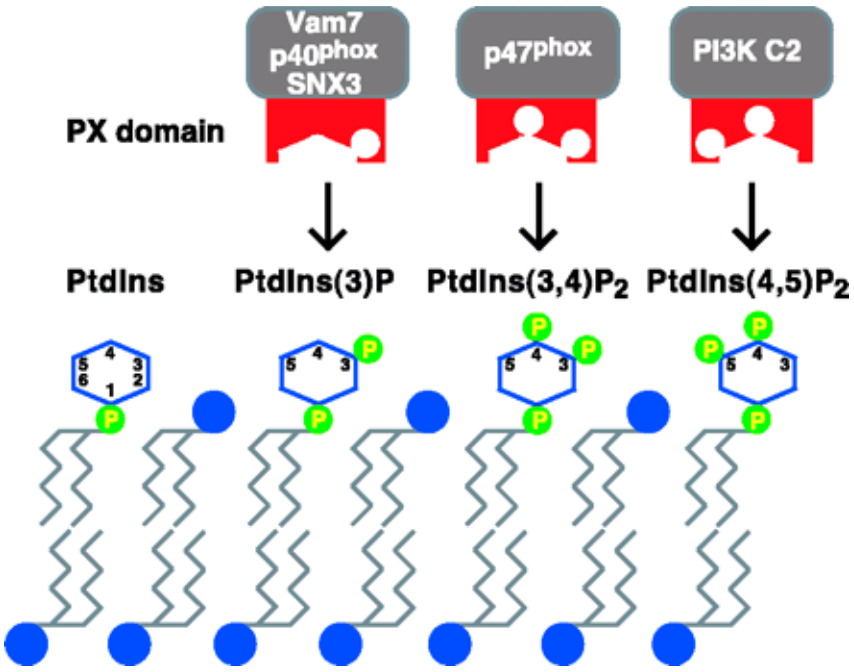

Figure 1.4.3 Schematic diagram of three types of PIs that bind to PX domains. PX domains predominantly bind with PI3P with high affinity, although there are reports which show the interaction between PX domains and PtdIns(4,5)P2 or PtdIns(3,4)P2 (Sato et al., 2001).

1.4.3 Function of Vps34

Vps34-Vps15 heterodimer is involved in different multiprotein complexes, which specify its elaborate biological roles. The biological functions of Vps34 include the regulation of vesicle trafficking including autophagy, endocytosis, phagocytosis and mTOR nutrition-sensing pathway (Fig. 1.4.4) (Backer, 2008; Vanhaesebroeck et al., 2010).

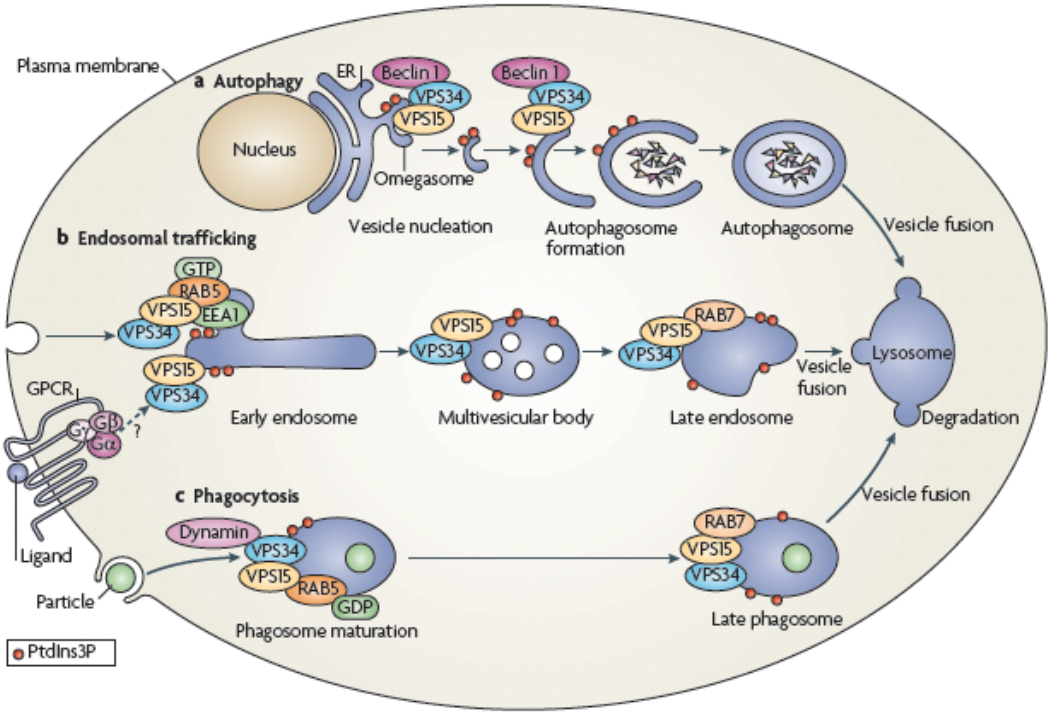

Figure 1.4.4 Functions of Vps34 in mammalian cells. Vps34 is actively involved in diverse vesicular trafficking events, such as autophagy (a), endosomal trafficking (b) and phagocytosis(c) (Vanhaesebroeck et al., 2010).

Autophagy is a special protein degradation process by which cytoplasmic components are transported to the lysosome for digestion (Levine and Klionsky, 2004; Klionsky, 2007). Convincing evidence has shown that the autophagic process is related to cell cycle regulation, starvation adaptation, ageing, and cancer development (Mathew et al., 2007; Mizushima et al., 2008). For autophagy, Vps34 was found in a multiprotein complex which also includes Vps15, Beclin1/Atg6, UVRAG, and Bif-1 in mammals and Vps15, Atg6, and Atg14 in yeast (Mari and

Reggiori, 2007). In yeast, this Vps34 complex plays a crucial role in recruiting autophagy-related (Atg) proteins to the preautophagosomal structure where autophagosomes assemble specifically (Suzuki et al., 2007). However, the role of Vps34 for autophagy in mammals is not well understood.

Vps34 was reported to be regulated by nutrients such as amino acids and glucose and by GPCRs (Byfield et al., 2005; Nobukuni et al., 2005; Slessareva et al., 2006). Several groups provide evidence that Vps34 can contribute to activation of mTORC1 in response to amino acid stimulation. Disruption of Vps34 activity with blocking antibodies or siRNA was found to block the activation of mTOR by amino acids and glucose, but not insulin (Byfield et al., 2005; Nobukuni et al., 2005). These findings indicate there is a link between Vps34 and mTORC1 which is a pivotal mediator of upstream metabolic signals to regulate cell growth and proliferation. However, Neufeld and colleagues got different result by showing that in *Drosophila melanogaster* Vps34 is not required for TOR activity. Mutation of Vps34 did not affect TOR-dependent phenotypes nor to interrupt TOR-dependent signaling. This finding indicated that possibly there is a fundamental difference in signaling mechanisms in different model systems (Juhász et al., 2008). In addition, these findings are inconsistent with the well-known role of Vps34 in promoting autophagy under nutrients-deficient conditions, suggesting that Vps34 is very likely to participate in many different regulation mechanisms. Together, there are still many open questions regarding the role of Vps34 in mTOR-mediated nutrient pathway. The involvement of Vps34 in mTOR signaling remains to be investigated.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Solutions and reagents

Reagents were obtained from the following sources: Insulin, 1-butanol (1-BtOH), and t-BtOH were from Sigma; wortmannin was from Calbiochem. DMEM (D5796), and DMEM lacking Arg, Leu, and Lys (D9463), were obtained from Sigma. 50X MEM amino acid solution without L-Gln (Sigma M5550) was used for stimulation by amino acids. For stimulation, the amino acid solution was added to a 2X concentration. Glucose-free DMEM was from Invitrogen (11966-025). PLD inhibitors for PLD1 (Compound14, VU0379595)[(1R,2R)-N-([S]-1-{4-[5-bromo-2-oxo-2,3-dihydro-1Hbenzo(d)-imidazol-1-yl]piperidin-1-yl}propan-2-yl)-2-phenylcyclopropane-carboxamide]; and PLD2 (Compound 22a, VU0364739) [N-(2-(1-(3- fluorophenyl) -4-oxo-1,3,8-Triazaspiro [4.5] decan-8-yl)ethyl)-2-naphthamide] were provided by Dr. H. Alex Brown (Vanderbilt University).

2.1.2 Cells lines

MDA-MB-231, Calu-1 and T24 cell lines were obtained from American Type Culture Collection (ATCC).

2.1.3 siRNA

siRNAs targeting RalA (M-009235-00-0005), Rheb (M-009692-02-0005), ARF1 (M-011580-01-0005), ARF6 (M-004008-01-0005), hVps34 (M-005250-00-0005), and hVps15 (M-005025-02-0005) were obtained from Dharmacon. Lipofectamine RNAiMAX for siRNA transfection was purchased from Invitrogen.

2.1.4 Antibodies

Antibodies against ARF1, ARF6, Rheb, and hVps15 were obtained from Santa Cruz Biotechnology; antibodies against S6 kinase, phosphorylated S6 kinase (Thr389), hVps34, RalA

and actin were obtained from Cell Signaling.

2.2 Methods

2.2.1 Cell culture conditions

All cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% bovine calf serum. The cells were incubated in a humidified chamber at 37ºC and 5% CO_2.

2.2.2 siRNA transfection

Cells were plated on 6-well plates at 20% confluence in medium containing 10% serum. After overnight, cells were transfected with siRNA at 80 nM concentration using Lipofectamine RNAiMAX (Invitrogen) according to manufacturer's instructions. After 24 hr, the media was changed to fresh media containing 10% serum and two days later cells were lysed and analyzed by western blot.

2.2.3 Western blot analysis

Proteins were extracted from cultured cells with 1% modified RIPA buffer (65 mM Tris-HCl (pH7.4), 154 mM NaCl, 1% IGEPAL, 0.25% Na-deoxycholate, 1 mM EDTA, 1mM activated sodium orthovanadate, 1 mM NaF, 1X Protease Inhibitor Cocktail, 1X Phosphatase Inhibitor Cocktail). 20µg whole cell lysate was then separated by SDS-PAGE and transferred onto nitrocellulose membranes. Western blot analysis of extracted proteins was performed using the ECL system (Thermo Scientific) as described in the manufacture's instruction. Relative levels of p70S6K phosphorylation were determined by measuring the intensity of the appropriate band in the autoradiograph with Image-J software.

2.2.4 PLD assay

PLD activity was determined by the transphosphatidylation reaction in the presence of

0.8% 1-BtOH as described previously. Cells in 60-mm culture dishes were labeled with [3H]-myristic acid (3.0 μCi) for 4-6 hrs and 1-BtOH (0.8%) was added 20 minutes before lipids were collected. Lipids were extracted and characterized by thin-layer chromatography as described previously. Relative levels of PLD activity were then determined by measuring the intensity of the corresponding phosphatidyl-butanol band in the autoradiograph with Image-J software or by scraping the thin layer chromatography plates and scintillation counting of the phosphatidyl-butanol band.

2.2.5 Immunoprecipitation

Cells were grown in 10 cm diameter plates. Immediately before lysing, culture plates were rinsed twice with cold PBS and lysed on ice for 20 min in 500 μl of ice-cold CHAPS IP buffer (40 mM HEPES [pH 7.5], 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 0.5 mM orthovanadate, and EDTA-free protease inhibitors [Roche]) containing 0.3% CHAPS. 0.5-1 mg protein was then incubated with appropriate antibodies and the immunoprecipitates were recovered 4-6 hrs later by using protein A or G sepharose beads. The immunoprecipitates were then subjected to Western blot analysis along with 20 μg of total cell lysate.

CHAPTER III

PLD IS REQUIRED FOR THE ACTIVATION OF MTORC1 PATHWAY BY NUTRIENTS

3.1 Introduction

The mammalian target of rapamycin (mTOR) is a pivotal regulator of cell growth, cell proliferation and cell survival. mTOR is activated in response to both growth factors and nutrients – most notably amino acids. While much is known about the activation of mTOR in response to growth factors, there is little known about the nutritional input into mTOR.

For the last decade, our lab and others were actively involved in deciphering the relationship between PLD and mTOR. Fortunately, we had been generating interesting and convincing data to prove that PLD and PA are critical upstream regulators for mTOR signaling. Fang et al. first reported that PA can physically associates with the FKBP12-rapamycin-binding (FRB) domain of mTOR. Consistent with data from Dr. Chen's group, our lab showed that elevated PLD activity in human breast cancer cells conferred rapamycin resistance which also indicates that PA and mTOR compete for binding with FRB domain of mTOR (Chen et al., 2003). Recently, our lab reported that PA, the metabolite of PLD, is required for the stability and activity of both mTORC1 and mTORC2 complexes (Toschi et al., 2009). Therefore, it will be very interesting to investigate the role of PLD in nutrition signaling pathway.

Although the amino acid sensor is still unknown, candidate mediators acting downstream of amino acids have been reported, including RalA GTPase, Rag GTPases, ARF GTPase, MAP4K3 and Class III PI3K catalytic subunit (also known as Vps34) (Nobukuri et al., 2005; Gulati et al., 2008; Yan et al., 2010). Interestingly, RalA has been reported to be required for amino acid induction of mTORC1 (Maehama et al., 2008). Our group also demonstrated that RalA is constitutively associated with PLD1 and recruits ARF family GTPases to activate PLD1 activity (Jiang et al., 1995; Luo et al., 1997; Luo et al., 1998). Another important study from Dr. Chen's group demonstrated that Rheb which is required for mTORC1 activation binds with PLD

and activates PLD1 activity (Sun et al., 2008). Moreover, there is evidence suggesting that maybe the link between amino acids and mTOR is the class III PI3K Vps34 (Byfiel et al., 2005; Nobukuni et al., 2005 2007; Gulati et al., 2008), which is an endosomal kinase that specifically produces the lipid PI3P (Backer, 2008). It is not known how Vps34 contributes to the activation of mTORC1, but PI3P serves to recruit proteins with PX domains (Backer, 2008). In this regard it is of interest that both PLD1 and PLD2 have PX domains (Morris, 2007) that could be influenced by Vps34 activity. Based on these studies, we hypothesized that some of these mediators act through PLD to further regulate the mTORC1 activity upon amino acids stimulation.

In this part, we report that amino acids do indeed stimulate PLD activity and that PLD activity is required for the activation of mTORC1 in response to amino acids. We further demonstrated that PLD-mTOR nutrition signaling pathway is dependent on RalA, ARF6 and Vps34. All these data reveal the critical role of PLD in mTOR-mediated nutrient signaling pathway.

3.2 Results

3.2.1 PLD activity is dependent on the availability of amino acids and glucose

PLD activity in several human cancer cell lines is elevated, especially those harboring Ras mutations including MDA-MB-231 breast, T24 bladder, and Calu-1 lung cancer cells (Zheng et al., 2007; Shi et al., 2007; Garcia et al., 2008). PA generated by PLD is required for the activation of mTORC1 (Fang et al., 2001; Toschi et al., 2009). Moreover, mTORC1 activity is regulated by nutritional sufficiency- including amino acids and glucose (Kim et al., 2002; Fingar and Blenis, 2004). We therefore examined whether the elevated PLD activity observed in human cancer cell lines was also affected by the availability of amino acids and glucose. The PLD activity in the MDA-MB-231, T24, and Calu-1 cells was evaluated in the presence and absence of essential amino acids (Fig. 3.2.1A) and glucose (Fig. 3.2.1B). As shown, the level of PLD activity in all three

cell lines was substantially reduced when the media lacked either amino acids or glucose. This result demonstrates that PLD activity in these cancer cell lines is regulated by nutrients.

3.2.2 mTORC1 activation induced by either amino acids or glucose is dependent on PLD activity.

As we already knew that mTORC1 activity is regulated by the availability of amino acids and glucose. Several laboratories demonstrated the specific correlation showed by the phosphorylation of mTOR substrates S6K1 and 4EBP1 due to amino acid stimulation, especially the branched chain amino acid leucine. Moreover, PA generated by PLD is indispensable for mTOR activation. We therefore examined whether amino acid-stimulated p70S6K phosphorylation in MDA-MB-231, T24 and Calu-1 cells was dependent on PLD activity. Cells were shifted to media without serum overnight and then the cells were placed in medium lacking

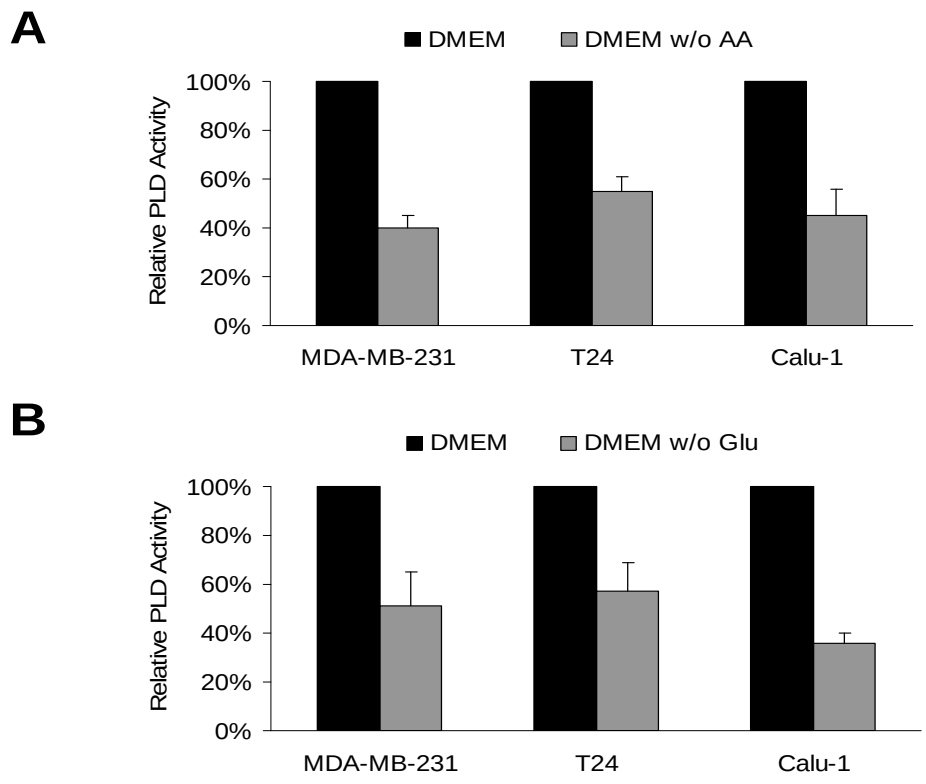

Figure 3.2.1 PLD activity is regulated by the availability of amino acids and glucose. MDA-MB-231, T24 and Calu-1 cells were plated at 40-50% confluence. After 24 hours, they were shifted to media containing 0.5% serum with either complete DMEM or DMEM lacking either essential amino acids (A) or glucose (B) for 4 hr along with 3H-myristic acid to label the lipids. 1-BtOH was added for 20 min before the cells were lysed and the level of PLD activity indicated by the transphosphatidylation product PtBt was determined as described in Materials and Methods. Relative PLD activity without either amino acids or glucose (black) was normalized to the levels of PLD in complete medium (DMEM) controls (grey). Data from at least two independent experiments are plotted as bar graph, and the error bars represent standard deviation.

both serum and essential amino acids. Four hours later amino acids were added in the presence of either 1-BtOH or t-BtOH and phosphorylated p70S6K at Thr389 was examined 30 min later. As we knew, 1-BtOH can suppress the production of PA by PLD via the transphosphatidylation reaction, t-BtOH can not participate in this reaction and is used as a negative control. As we can see, 1-BtOH, but not t-BtOH, inhibited the increase in phosphorylated p70S6K induced by amino

acid stimulation in all three cell lines (Fig. 3.2.2A). These data confirm that the induction of mTORC1 by amino acids is dependent on PLD activity. At the same time, we also examined the PLD dependence for the activation of mTORC1 by glucose. MDA-MB-231, T24, and Calu-1 cells were placed in medium lacking serum overnight and then shifted to media lacking both serum and glucose. Four hours later glucose was added along with either 1-BtOH or t-BtOH and the phosphorylation of p70S6K was also evaluated 30 min later. As shown in Fig. 3.2.2B, the

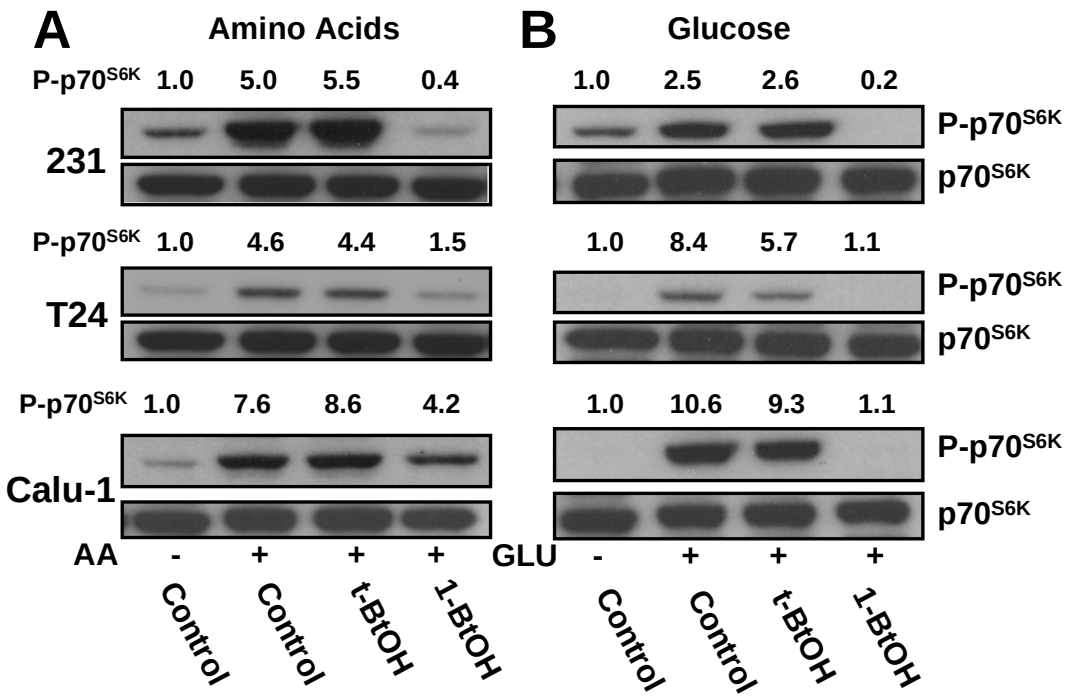

Figure 3.2.2 mTORC1 activation induced by either amino acids or glucose is dependent on PLD activity. MDA-MB-231, T24 and Calu-1 cells were first cultured in DMEM with 10% FBS. The cells were then starved in DMEM without serum overnight. Later the cells were shifted to medium lacking either essential amino acids (A) or glucose (B). 4 hr later, amino acids or glucose were added back in the presence of either 1-BtOH or t-BtOH as indicated. Cells were harvested 30 min later and the level of phosphorylated $p70^{S6K}$ at Thr389 ($P\text{-}p70^{S6K}$) was examined by Western blot. The levels of P-p70S6K relative to the level of $p70^{S6K}$ were determined by ImageJ and normalized to the level observed in the absence of amino acids (A) or glucose (B) and are provided above each blot. The data shown are representative experiments repeated at least twice.

induction p70S6K phosphorylation was suppressed by 1-BtOH, but not by t-BtOH.

Since there is always controversy about the specificity of 1-BtOH to inhibit PLD activity, we further examined the effect of the PLD inhibitors on the amino acid- and glucose-induced increases in p70S6K phosphorylation. We tested the efficiency of PLD1 and PLD2 specific inhibitors, Compounds 14 and 22a, to inhibit the PLD activity in all three cell lines. As we can see from Fig. 3.2.3, both inhibitors can significantly suppress PLD activity more than 50%. We

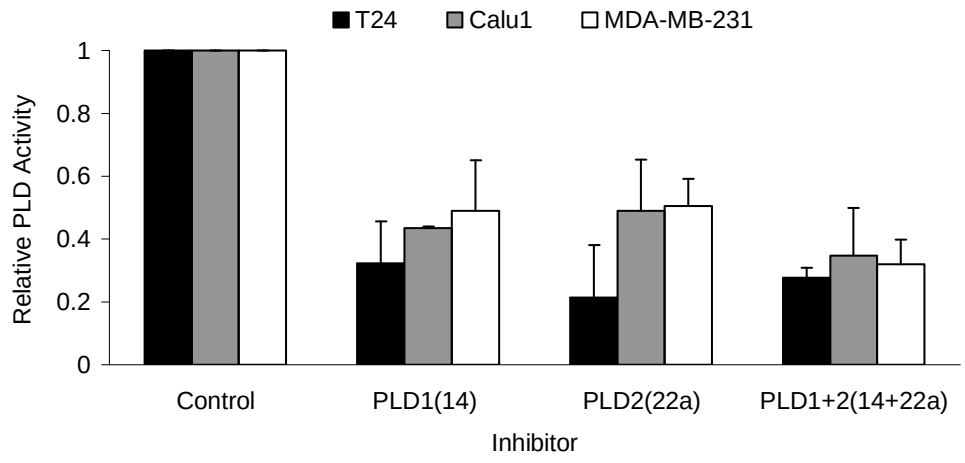

Figure 3.2.3 Amino acids and glucose dependent PLD activity is inhibited by PLD inhibitors. T24, Calu-1 and MDA-MB-231 cells were cultured in 6-well plates. The cells were shifted to medium lacking serum for 4 hr. The PLD1 inhibitor Compound 14 and the PLD2 inhibitor Compound 22A were added at the concentrations indicated for one hour, at the same time the cells were labeled with 3H-myristate and the relative PLD activity was then determined as before by PLD assay. Data for T24 (black), Calu-1 (grey) and MDA-MB-231 (white) cells from at least two independent experiments are plotted as bar graph, and the error bars represent standard deviation.

further checked the effect for these inhibitor on mTORC1 activity induced by amino acids and glucose. As shown in Fig. 3.2.4, the combination of Compounds 14 and 22a suppressed the induction of p70S6K phosphorylation by both amino acids and glucose in the T24 cells. Neither of the inhibitors alone had a significantly stronger impact of p70S6K phosphorylation (data not shown) – making it likely that both PLD1 and PLD2 are involved. These further prove that PLD

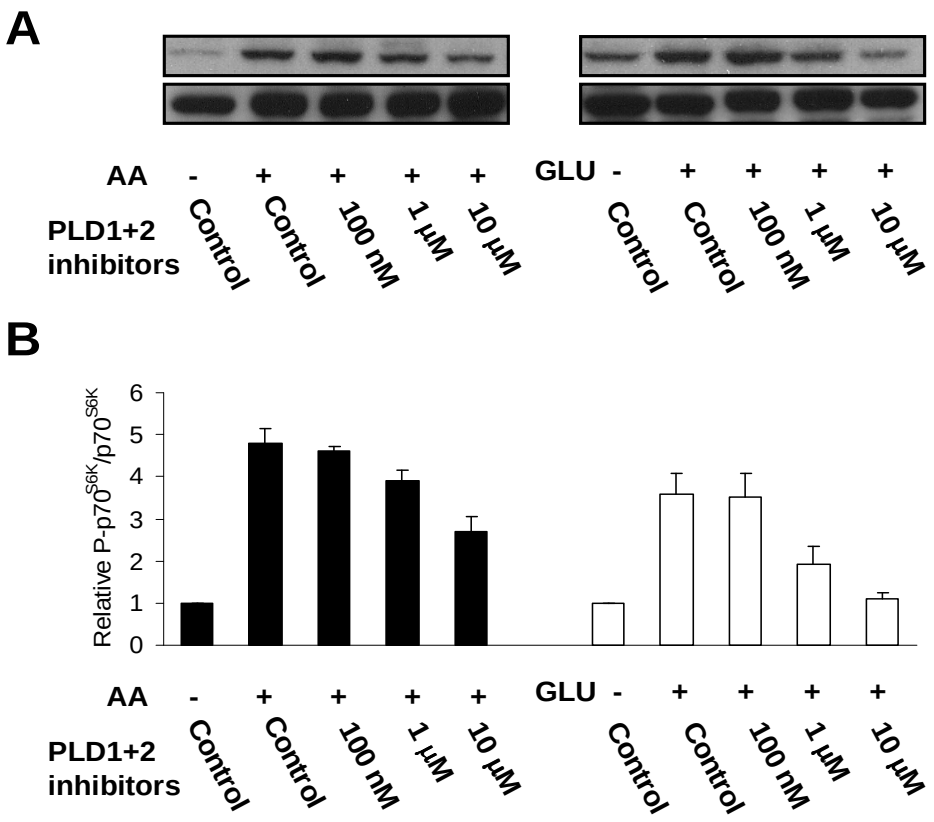

Figure 3.2.4 Amino acid- and glucose-induced increases in mTORC1 activity are suppressed by PLD inhibitors. T24 cells were cultured in 6-well plates. After overnight serum starvation and later 4 hr amino acids or glucose starvation, amino acids or glucose were added back in the presence of the indicated concentration of the PLD inhibitors 14 and 22A. P-p70^{S6K} was evaluated 30 min later by western blot (A). Bar graphs (B) are based on at least two independent experiments, and the error bars represent standard deviation.

activity is required for the induction of mTORC1 with either glucose or amino acids, indicating that PLD possibly plays a significant role in nutrient signaling pathway.

3.2.3 PLD activity and mTORC1 activity induced by either amino acids or glucose in human cancer cells are dependent on Rheb

Recently Sun et al. reported that Rheb can bind to and stimulate the activity of PLD1 in a GTP-dependent manner by in vitro assay. Rheb is therefore proposed another important upstream regulator of PLD (Sun et al., 2008). We therefore examined whether the amino

acid-dependent PLD activity in human cancer cells was dependent on Rheb. As shown in Fig. 3.2.5A, suppression of Rheb expression with siRNA suppressed the amino acid dependent PLD activity in MDA-MB-231, T24, and Calu-1 cells. Consistent with previous reports (Nobukuni et al., 2005), knockdown of Rheb also suppressed the amino acid-induced phosphorylation of p70S6K phosphorylation at the mTORC1 Thr389 site (Fig. 3.2.5B). We also examined the impact of suppressing Rheb expression on the stimulation of mTORC1 with glucose in the T24 cells. As shown in Fig. 3.2.5C, the glucose-induced increase in p70S6K phosphorylation was also dependent on Rheb. These data demonstrate that nutrient-dependent PLD activity is dependent on Rheb and that the nutrient induction of mTORC1 is also dependent on Rheb.

3.2.4 Amino acid signaling is independent of AKT-TSC regulation

Since it was demonstrated that amino acid induction of mTORC1 is dependent on Rheb, therefore it is very possible that mTORC1 activation induced by amino acids is also dependent on the suppression of TSC1/2, which functions as GAP for Rheb GTPase and suppresses Rheb by stimulating the hydrolysis of GTP to GDP (Inoki et al., 2003). We therefore investigated the impact of amino acids on the phosphorylation state of TSC2, which is both activated and suppressed by phosphorylation at different sites (Inoki et al., 2002; Manning et al., 2002; Ma et al., 2005). Akt phosphorylates TSC2 at Thr1462, which leads to the disruption of TSC1/2 complexes and the suppression of the GTPase activity of Rheb (Manning et al., 2002). As shown in Fig. 3.2.6A, amino acids had no effect on the phosphorylation state of TSC2 at Thr1462 in either the MDAMB-231 or T24 cells. Suppression of PA generation by PLD with 1-BtOH also had no effect on the phosphorylation of TSC2 at this site (Fig. 3.2.6A). In contrast, insulin was able to increase phosphorylation at this site (Fig. 3.2.6B). TSC2 is phosphorylated by Akt, which is activated by PI3K. Importantly, suppression PI3K with inhibitor wortmannin was able to

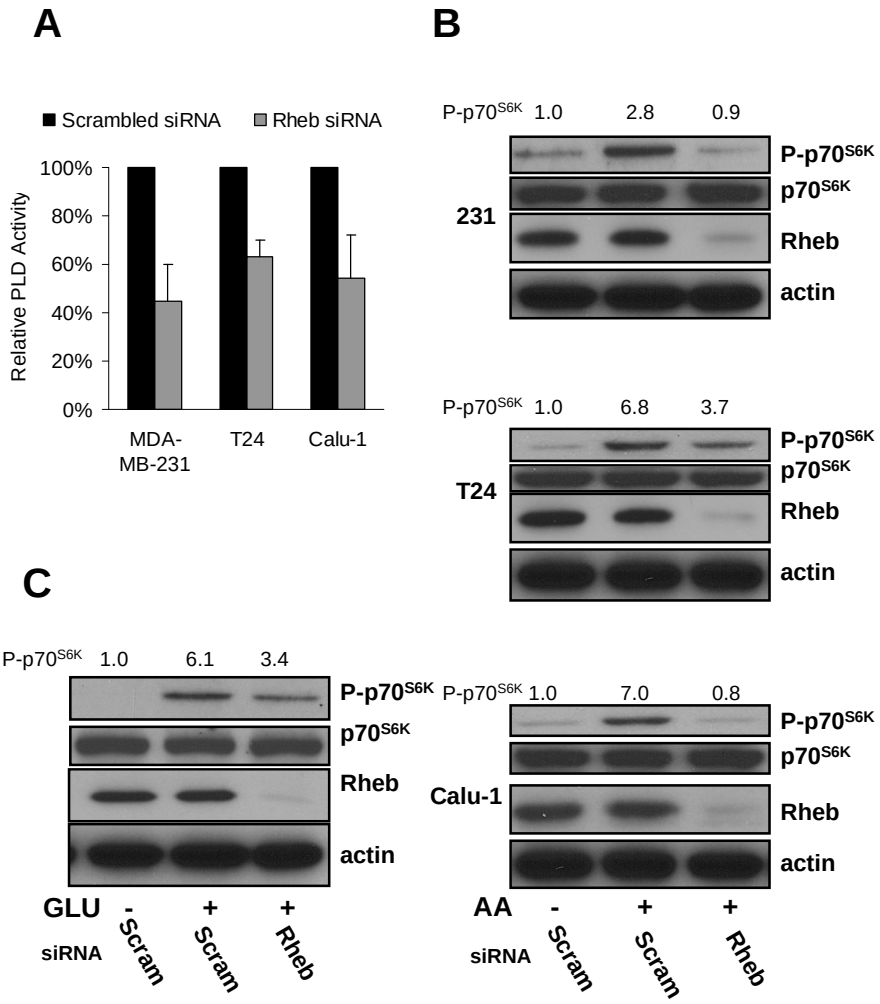

Figure 3.2.5 PLD activity and mTORC1 activity induced by either amino acids or glucose in human cancer cells are dependent on Rheb. (A) MDA-MB-231, T24 and Calu-1 cells were transfected with Rheb siRNA or a scrambled (Scram) control siRNA. 48 hr later the cells were shifted to medium with 0.5% serum overnight. The cells were then harvested and the PLD activity was determined by PLD assay. Data from at least two independent experiments are plotted as bar graph, and error bars represent standard deviation. (B) Cells were first transfected with Rheb or scrambled siRNA as in A. After overnight serum starvation, the cells were placed in medium without essential amino acids for 4 hr. Then amino acids were added back for 30 min and P-p70^{S6K} was determined by Western blot. (C) T24 cells were treated as in (B) with Rheb or scrambled siRNA and then put in medium without glucose for 4 hr. Glucose was then added as indicated and the levels of P-p70^{S6K}, p70^{S6K}, Rheb and actin were determined 30 min later by Western blot analysis. The data shown are representative experiments repeated at least twice.

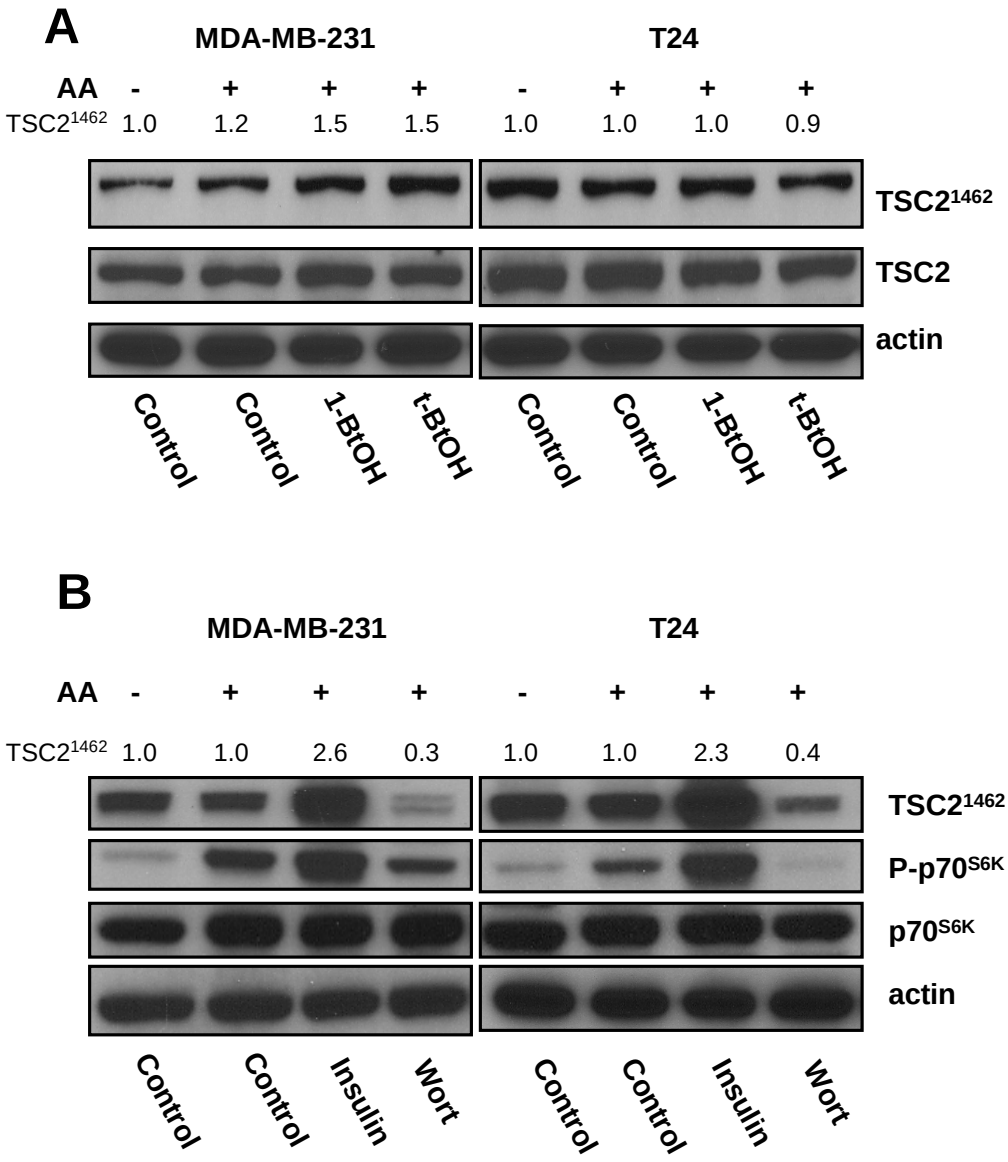

Figure 3.2.6 Amino acid signaling is independent of AKT-TSC regulation. (A) MDA-MB-231 and T24 cells were plated in 6-well plate and then shifted to medium without serum overnight. The cells were then shifted to medium without essential amino acids for 4 hr. As indicated, amino acids were added back in the presence of either 1-BtOH or t-BtOH for 30 min and TSC2 phosphorylation was then determined by Western blot using an antibody specific for TSC2 phosphorylated at Thr1462. (B) MDAMB-231 and T24 cells were prepared and treated as in (A). Where indicated, amino acids were added back in the presence of either insulin or wortmannin for 30 min and TSC2 phosphorylation was then determined.

suppress TSC2 phosphorylation at Thr1462 and this led to reduced phosphorylation of p70S6K

at Thr389. Taken together, the data in Fig 3.2.5 and Fig 3.2.6 indicate that while signaling through TSC and Rheb are required for the amino acid-induction of PLD and mTORC1, this signaling pathway is not impacted by amino acids. These findings are consistent with previous reports indicating that nutrient signaling to mTOR does not upregulate the TSC-Rheb pathway (Nobukuni et al., 2005; Smith et al., 2005).

3.2.5 RalA is required for PLD activity and nutrient induced mTORC1 activation

The findings that amino acids signaling to mTORC1 is independent of AKT-TSC pathway suggest that the amino acid-stimulated increase may be acting through other upstream regulators of PLD to further modulate mTORC1 activity. It was reported that the GTPase RalA is required for the amino acid-induced activation of mTORC1 (Maehama et al., 2008). The RalA dependence for the amino acid dependent activation of mTORC1 is particularly intriguing in that RalA is constitutively associated with PLD1 and is required for the activation of PLD1. To test the role of Ral-PLD pathway for nutrition signaling, we knocked down RalA with siRNA and the impact on amino acid dependent PLD activity was evaluated in the MDA-MB-231 and T24 cells. As shown in Fig. 3.2.7A, the amino acid-dependent PLD activity in both cell lines was substantially suppressed with the depletion of RalA which confirmed the importance of RalA for PLD activity. We next examined the effect of RalA knockdown on the induction of p70S6K phosphorylation by amino acids. As shown in Fig. 3.2.8B, RalA knockdown suppressed the amino acid induction of p70S6K phosphorylation at the mTORC1 site at Thr389 in suppressed by RalA knockdown in the T24 cells. In contrast, the serum induction of p70S6K phosphorylation was not impacted by RalA knockdown in these cells (Fig. 3.2.5C). These data are consistent with RalA being a key target of nutrients for the activation of mTORC1 and further implicate PLD1, which interacts directly with RalA as a conduit to mTORC1 activation

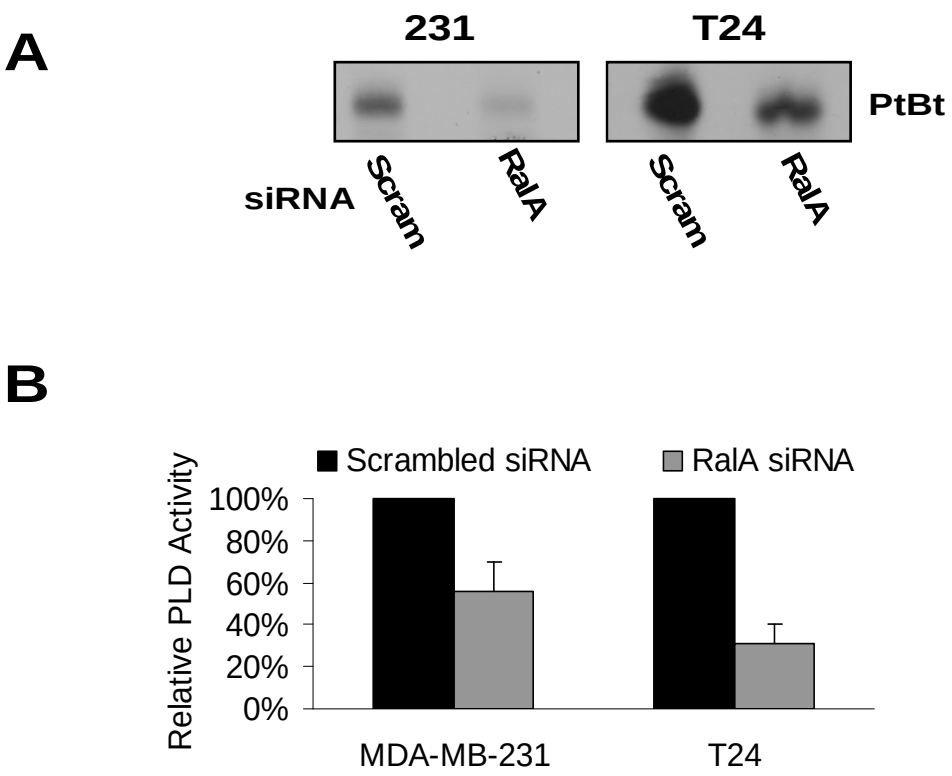

Figure 3.2.7 PLD activity is dependent on RalA. (A) MDA-MB-231 and T24 cells were transfected with RalA siRNA or scrambled (Scram) control siRNA when they reach 30% confluence. The cells were then harvested 72 hours later and the PLD activity was determined by PLD assay. (B) Data from at least two independent experiments are plotted as bar graph, and the error bars represent standard deviation. The relative PLD activity with RalA siRNA (grey) is plotted as percent of basal PLD activity with scrambled siRNA (black) which is given the value of 100%.

T24 cells. The induction of p70S6K phosphorylation by glucose was similarly.

3.2.6 Amino acid and glucose-induced mTORC1 activity and PLD activity are dependent on ARF6

Our lab previously reported that RalA stimulates the activation of PLD1 by recruitment of ARF family GTPases into a RalA-PLD1 complex (Liu et al., 1997; Liu et al., 1998). We also demonstrated that HRas-induced PLD activity was dependent on ARF6 (Xu et al., 2003). ARF6 has also been implicated in the glucose stimulation of insulin secretion (Ma et al.,

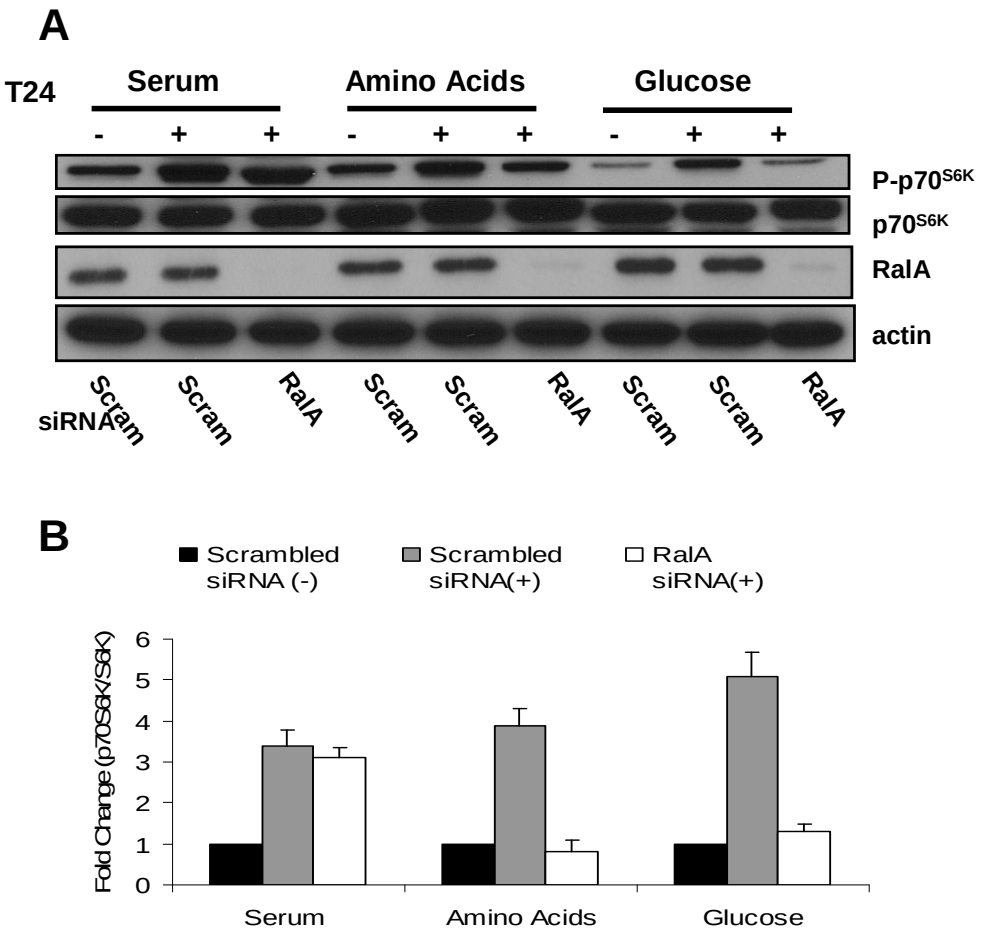

Figure 3.2.8 mTORC1 activation induced by amino acids is dependent on RalA. T24 cells were transfected with RalA or scrambled siRNA. 4 hr before collecting the cell lysate, cells were put in medium without serum (all lanes) and either amino acids or glucose. Serum, amino acids and glucose were then added as indicated and the levels of p-p70^{S6K}, p70^{S6K}, RalA and actin were determined 30 min later by Western blot analysis. (B) The levels of P-p70^{S6K} relative to p70^{S6K} from at least two independent experiments were determined and plotted as bar graph, and the error bars represent standard deviation.

2010)–indicating that ARF6 is activated in response to nutrients. Thus, the involvement of RalA, in the amino acid- and glucose-dependent PLD activity shown in Fig. 3.2.7 suggests the possible involvement of ARF GTPases, which are direct activators of PLD1 (Exton, 2002). As reported previously for the MDA-MB-231 cells (Garcia et al., 2008), knockdown of either ARF1 or ARF6 with siRNA suppressed the nutrient-dependent PLD activity in the T24 cells (Fig. 3.2.9A).

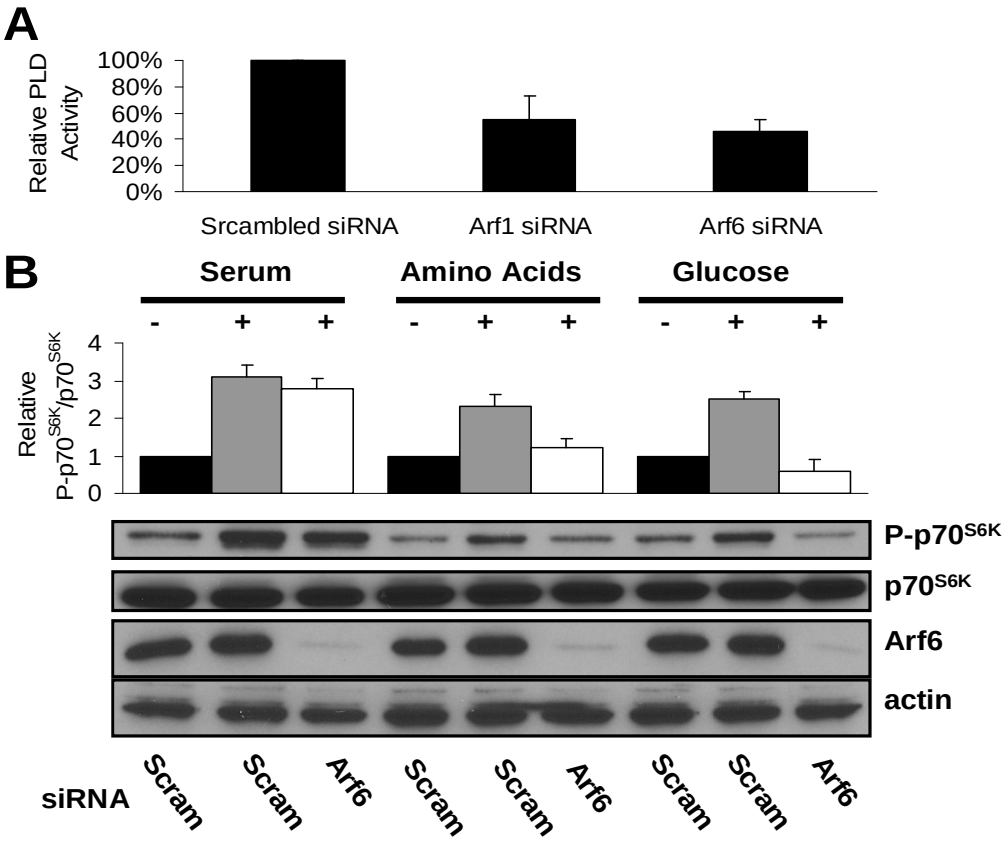

Figure 3.2.9 ARF6 is required for PLD activity and mTORC1 activation induced by nutrients. (A) T24 cells were transfected with either ARF6 or ARF1 siRNAs or scrambled (Scram) siRNA as indicated and then put in media lacking serum overnight. PLD activity was evaluated as described before. Values were normalized to the control scrambled siRNAs, which were considered a value of 100%. (B) T24 cells were treated as in (A) with either ARF6 or scrambled (Scram) siRNA controls and then put in medium lacking serum (all lanes) and either amino acids or glucose as indicated for 4 hr. Serum, amino acids and glucose were then added as indicated and the levels of P-p70^{S6K}, p70^{S6K}, ARF6, and actin were determined 30 min later by Western blot analysis. The levels of P-p70^{S6K} relative to p70^{S6K} from at least two independent experiments were determined and plotted as bar graph, and the error bars represent standard deviation.

As shown in Fig. 3.2.8A, ARF6 knockdown suppressed the induction of p70S6K phosphorylation by both amino acids and by glucose in the T24 cells. As with RalA, the serum induction of p70S6K phosphorylation was not impacted by ARF6 knockdown. In contrast with

ARF6, knockdown of ARF1 did not affect the stimulation of p70S6K phosphorylation by amino acids, glucose, or serum (Fig. 3.2.10). These data are consistent with ARF6 being a key target of nutrients for the activation of mTORC1 and further implicate PLD1, which interacts directly with RalA as a conduit to mTORC1 activation.

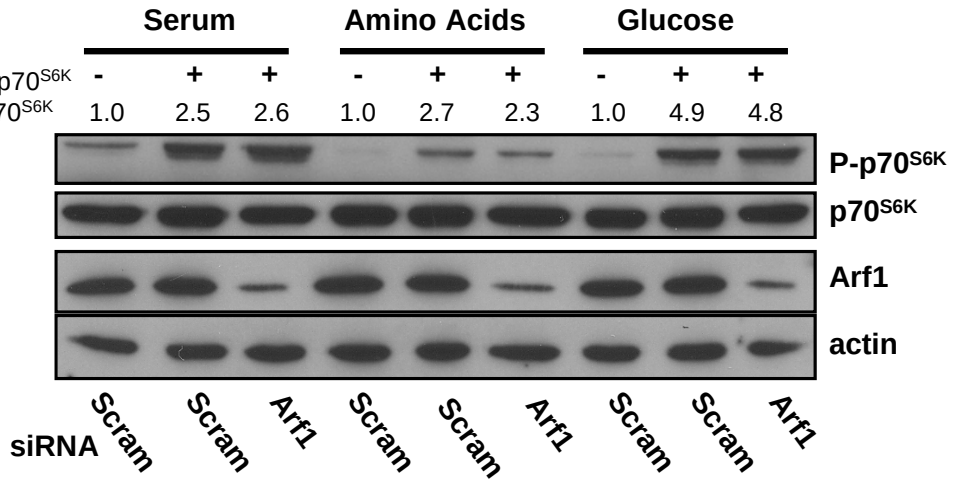

Figure 3.2.10 mTORC1 activation induced by either amino acids or glucose is independent of ARF1. T24 cells were transfected with either ARF6 or scrambled (Scram) siRNA as indicated. After 48 hr, the cells were serum starved overnight, then put in medium lacking serum (all lanes) and either amino acids or glucose as indicated for 4 hr. Serum, amino acids and glucose were then added back as indicated and the levels of P-p70^{S6K}, p70^{S6K}, ARF6, and actin were determined 30 min later by Western blot analysis. The levels of P-p70^{S6K} relative to p70^{S6K} were determined by ImageJ.

3.2.7 Class III PI3K is required for amino acid-dependent PLD activity

Several reports proposed that type III PI 3-kinase functions as a signaling intermediate in amino acid-stimulated activation of mTORC1 (Nobukuni et al., 2005; Byfield et al., 2005). hVps34 is the sole member of class III PI3K kinase and works together with hVps15, the regulatory subunit, to generate PI3P. As mentioned before, PI3P can specifically interact with proteins with PX domains -and interesting, both PLD1 and PLD2 have a PX domain that is critical for its activity (Frohman et al., 1999; Stahelin et al., 2004), therefore it will be very

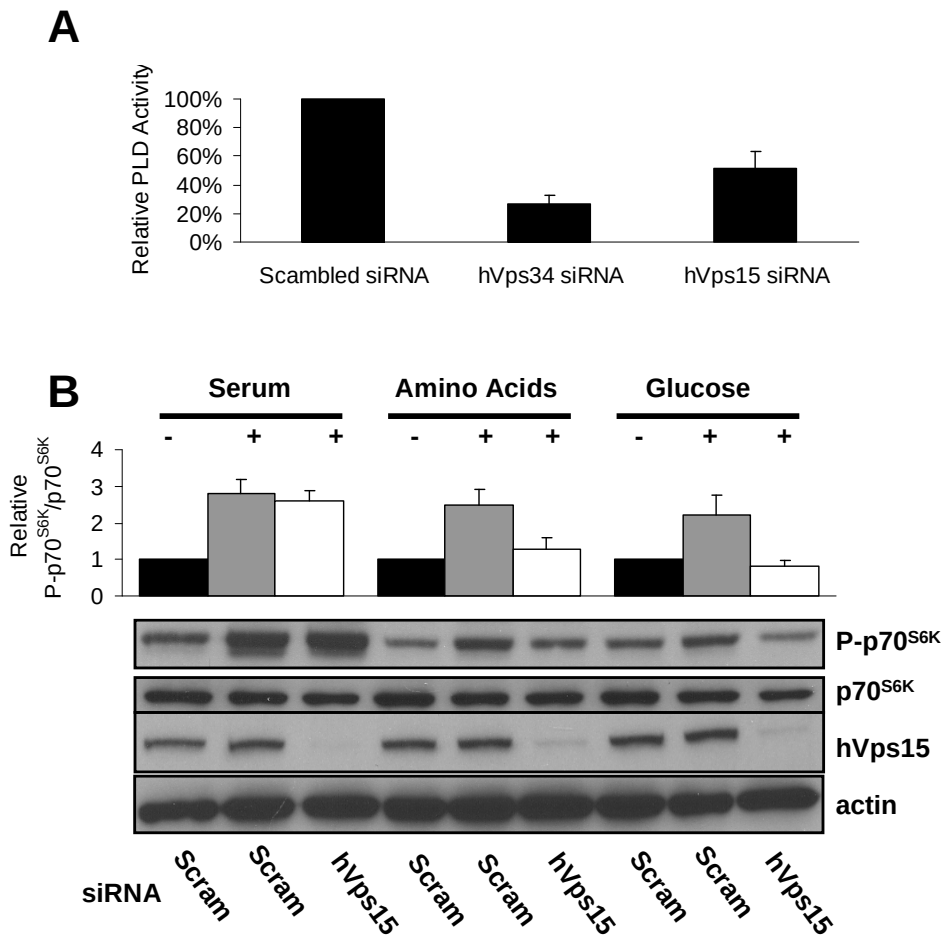

Figure 3.2.11 PLD activity and nutrients-stimulated mTORC1 activity are regulated by Vps15. (A) MDA-MB-231 and T24 cells were transfected with hVps34, hVps15, or scrambled control siRNA as indicated. 72 hr later, the cells were harvested and the relative PLD activity was determined as described in Materials and Methods. Values were normalized to the control scrambled siRNAs, which were given a value of 100%. (B) T24 cells were treated with either hVps15 or scrambled siRNA and then put in media lacking serum (all lanes) and either amino acids or glucose as indicated for 4 hr. Serum, amino acids and glucose were then added where indicated and the levels of p-p70^{S6K}, p70^{S6K}, hVps15, and actin were determined 30 min later by Western blot analysis. The levels of P-p70^{S6K} relative to p70^{S6K} from at least two independent experiments were determined and plotted as bar graph, and the error bars represent standard deviation.

interesting to investigate if PLD activity is regulated by hVps34. We therefore examined whether hVps34 was required for the amino acid dependent PLD activity in T24 cells. Knockdown of

hVps34 with siRNA significantly inhibited the amino acid-dependent PLD activity in T24 cells. At the same time, we also examined the effect of suppressing hVps15 (formerly known as P150) on PLD activity in the T24 cells. hVps15 is required for catalytic activity of hVps34 and has also been implicated in nutrient sensing (Nobukuni et al., 2005; Yan et al., 2009). As shown in Fig. 3.2.11A, PLD activity dramatically decreased with the depletion of hVps15 by siRNA.

These data are consistent with our previous finding that the PI3K inhibitor LY294002 strongly inhibited PLD activity in the T24 and Calu-1 cells (Shi et al., 2007). We next examined whether the knockdown of hVps34 suppressed the induction of p70S6K phosphorylation by serum, amino acids and glucose. We were unable to detect significant effects upon the phosphorylation of p70S6K in the T24 cells (data not shown). However, since the hVps34 has been shown previously to be required for amino acid-induced increases in mTORC1 activity, we examined the effect of suppressing the hVps15. As shown in Fig. 3.2.11B, suppression of hVps15 expression suppressed both the amino acid and glucose induction of p70S6K phosphorylation in T24 cells. It is still unknown at this point why the depletion of hVps15 was more effective than depletion of hVps34, but the effect of hVps15 and hVps34 siRNAs on the amino acid- and glucose-dependent PLD activity suggest that the signals generated by hVps34 that lead to mTORC1 go through PLD. This finding further supports the critical role of PLD in mTOR mediated nutrient signaling.

3.3 Discussion

It has been well known that mTORC1 regulates cell growth and proliferation in response to nutrient availability, growth factors, energy status. Whereas growth factors stimulation of mTORC1 is largely regulated through the PI3K/Akt/TSC/Rheb pathway, this signaling pathway is not impacted by nutrients (Nobukuni et al., 2005; Gulati and Thomas, 2007; Avruch et al., 2009). Growth factors modulate mTORC1 activity by modulating the activity of the tuberous sclerosis complex, the Rheb GTPase activating protein, to control Rheb GTP-GDP status (Zhang et al., 2003; Kwiatkowski and Manning, 2005). In contrast, the mechanism by which amino acids stimulate the mTORC1 activity remains to be fully defined. Therefore, our findings reveal a novel PLD dependent mechanism for activating mTORC1 in response to nutrients

The results from this study provide several lines of evidence to support the hypothesis that nutrients stimuli activate mTOR signaling through a PLD-dependent manner. Even though nutrients do not impact on Rheb GTP loading (Nobukuni et al., 2005; Long et al., 2005), Rheb is clearly required for the nutrient-dependent increase in mTORC1. In this study, we proved that Rheb was required for the nutrient dependent PLD activity, which is consistent with the data from Chen's group that revealed the role of Rheb as an important PLD upstream regulator by directly interacting with PLD1 to regulate its activity (Sun et al., 2008). Moreover, our study showed that the activation of nutrient-dependent mTORC1 activity was also dependent on the GTPases RalA and ARF6. Both of these GTPases have been implicated in both responding to nutrients and the stimulation of PLD activity. RalA is constitutively associated with PLD1, but does not activate PLD1 by itself. RalA contributes to the activation of PLD1 by recruiting ARF6, which does activate PLD1 activity, into RalA/ARF6/PLD1 complex (Luo et al., 1998). While it is still unknown from this study how the presence of nutrients activates RalA and ARF6, the data

provided here indicate that a key target of RalA and ARF6 for the stimulation of mTORC1 is PLD1. Therefore these findings contribute significantly to our better understanding of how signals from nutrient status were transmitted to mTOR to further coordinate cell growth and proliferation.

Even though the involvement of RalA and ARF6 in the nutrient-dependent increased in PLD and mTORC1 activity implicates the significance of PLD1, the role of PLD2 for mTORC1 activation should not be neglected. PLD2 has been implicated by several groups in the regulation of mTORC1. Exogenously expressed PLD2 was shown to increase p70S6K phosphorylation in different cell lines (Lehman et al., 2007; Frondorf et al., 2010). It was also reported that PLD2 directly interacts with mTOR and Raptor and this association was indispensable for mitogen stimulated mTORC1 activation (Ha et al., 2006). More recently, dominant negative mutants of both PLD1 and PLD2 were able to suppress the activation of mTORC1 (Toschi et al., 2009). Data from this study also reveal that the nutrient-stimulated mTORC1 activity and PLD activity were sensitive to PLD inhibitors specific for both PLD1 and PLD2. Taken together, it is very possible that both PLD1 and PLD2 participate in mTORC1 mediated nutrient signaling pathway.

mTOR has been reported to localize in many intracellular sites, including the Golgi apparatus, endoplasmic reticulum (ER), mitochondria, nucleus and the cytoplasm (Li et al., 2006; Aronova et al., 2007). It is very possible that diverse mTOR functions could involve the targeting of mTOR complexes to different subcellular compartments, thereby interacting with different sets of substrates to regulate a variety of cellular activities. Recently sabatini and colleagues found in cells stimulated with amino acids, mTOR is more likely to localize in the perinuclear region which overlapped with Rab7, implying that possibly mTOR translocated to the late endosomal and lysosomal compartments after nutrient stimulation. It is of particular interest that

Rab7 also interacts with hVps34/hVps15 heterodimer, therefore facilitating the recruitment of proteins with PX domains. It has been well known that both PLD1 and PLD2 contain this PX domain which is also essential for their localization and catalytic activity. In this regard, the mechanism by which hVps34/hVps15 complex activates mTOR activity in response to nutrient stimuli is that PLD is recruited to endosome or lysosome by interacting with PI3P generated by hVps34 and further activates mTORC1. Meanwhile, Rag GTPases associate with the mTORC1 protein kinase complex and translocate them to similar compartments where Rheb also resides (Sancak et al., 2008; Sancak and Sabatini, 2009). ARF6, but not ARF1 was also reported to localize in endosome where it will directly bind to and activate PLD activity (Hiroyama and Exton, 2005; Jovanovic et al., 2006). By recruiting all these regulators to specific endomembrane system of the cell, mTOR now can be activated upon amino acid stimulation. Interestingly, Neufeld and colleagues reported that in *Drosophila,* Vps34 is not required for amino acid stimulated mTORC1 activation even though it can still regualte autophagy and endocytosis (Juhasz et al., 2008). Based on our data, it is very possible that PLD is not required for mTOR activation in this model system. Indeed Chen and colleagues reported that *Drosophila* TOR lacks the Arg at 2109 that is critical for PA binding to mTOR, so that most likely mTOR can not be regulated by PLD and PA in *Drosophila* (Sun and Chen, 2008). Taken together, it appears that hVps34 plays a critical role in recruiting PLD to activate mTOR1 in nutrient stimulated cells.

Compared to amino acid signaling, much less is known how the level of glucose link to mTORC1 activity. Ma et al. recently reported that in pancreatic Beta cells high glucose induced insulin secretion through a ARF/PLD1 dependent pathway. In this study, we demonstrated that PLD also mediates the response of mTORC1 to glucose. It has not yet been demonstrated whether glucose, like amino acids would stimulate the Rag-mediated translocation to lysosomal

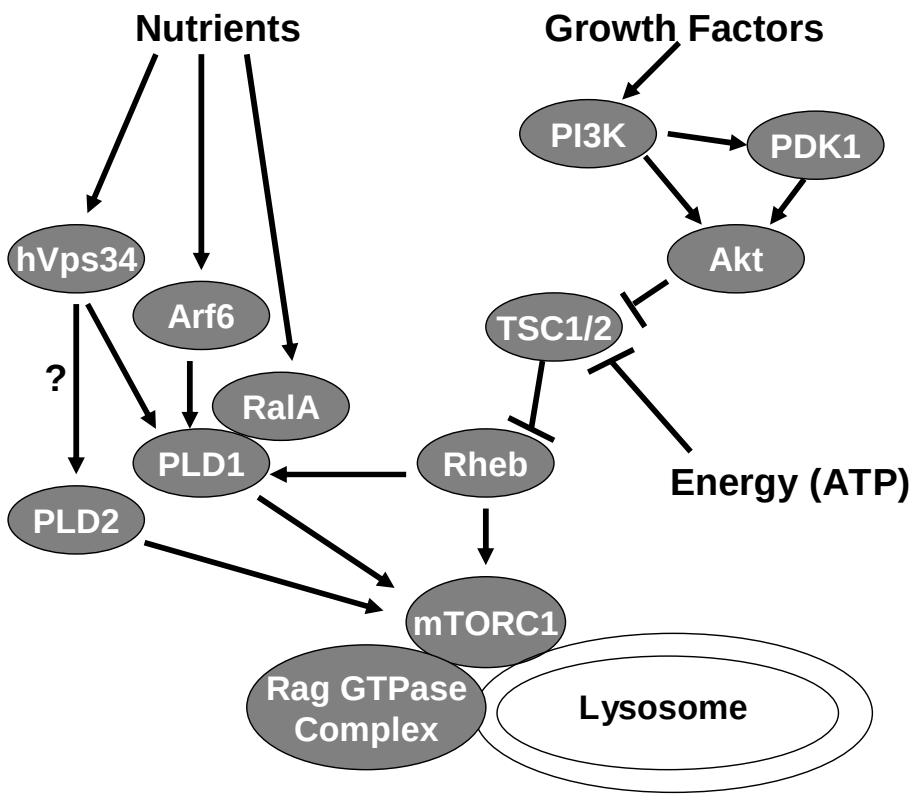

Figure 3.2.12 Model for mTOR mediated nutrient signaling. mTOR mediates two major inputs- one is from growth factors, another is from nutrients. The growth factors keep Rheb in a GTP-bound state through suppression of the tuberous sclerosis complex – TSC. Amino acids stimulate the recruitment of mTORC1 via Rag GTPases to lysosomal membranes where nutrients stimulate the activity of mTORC1 via the activation of PLD1 and possibly PLD2 by the activation of RalA, ARF6, and class III PI3K. Interestingly, Rheb is also required for PLD activity – revealing crosstalk between the two inputs into mTORC1. activation.

membranes. It is possible that glucose might target mTORC1 to a specific compartment where glucose metabolism is more related. Collectively, our study takes into account previous studies linking the nutrient response of mTORC1 to RalA, ARF6, and hVps34. Interestingly, all these factors are closely associated with PLD which further supports PLD as a central mediator for nutrient signaling. The findings presented in this study support the model that mTOR responds to growth factors and nutrients through distinct pathways. While growth factors activate mTORC1 through controlling Rheb GTP charging status mediated by PI3K signaling, and nutrients

stimulate mTORC1 activity by regulating PLD activity and the generation of PA. A model for the activation of mTORC1 by growth factors and nutrients is shown in Fig. 3.2.12. More importantly, the data presented in this study was obtained in human cancer cells with elevated PLD activity. PLD is considered oncogene since it provides a survival signal in a variety of cancer cell lines. The newly established role of PLD in nutrient signaling further highlight the significance of PLD in cancer, where cells need to deal with low oxygen, nutrient and growth factor environments for proliferation and survival. Therefore, the finding reported here that the PLD functions as an important mediator of nutrient signaling provides another support for targeting the survival signals generated by PLD in cancer cells.

Summary

It is well known that mTOR is a critical regulator for cell growth, cell proliferation and cell survival. mTOR responds to the signals from both growth factors and nutrients – most notably amino acids. Although much is known about the activation of mTOR in response to growth factors, there is much less known about the nutritional input into mTOR. Although Rheb is believed to be indispensable for mTORC1 activation in response to the stimulation of growth factors and nutrients, it is still controversial weather amino acid availability influences the nucleotide binding status of Rheb. TSC1/2 is a well-defined mediator for mTORC1 activation by mitogenic stimuli, however it is believed to be dispensable for amino acid-stimulated mTORC1 activation.

In this study we revealed a novel PLD dependent mechanism for activating mTORC1 in response to nutrients. mTOR plays critical roles in the regulation of cell growth, cell proliferation and cell survival. mTOR has also been implicated in the regulation of autophagy, which occurs in response to nutritional stress. mTOR is activated in most if not all cancer cells (Foster, 2009)

and suppression of mTOR has been correlated with extended lifespan (Kapahi et al., 2010). Dietary restriction, which has also been correlated with extended lifespan (Bordone and Guarente, 2005), leads to the suppression mTORC1 (Katewa and Kapahi, 2010). Thus, this study has the potential to have a broad impact of a variety of factors related to both aging and cancer.

CHAPTER VI

References

Abraham, R.T. (2002). Identification of TOR signaling complexes: more TORC for the cell growth engine. Cell 111, 9-12.

Ago, T., Takeya, R., Hiroaki, H., Kuribayashi, F., Ito, T., Kohda, D., and Sumimoto, H. (2001). The PX domain as a novel phosphoinositide- binding module. Biochemical and biophysical research communications 287, 733-738.

Antonescu, C.N., Danuser, G., and Schmid, S.L. (2010). Phosphatidic acid plays a regulatory role in clathrin-mediated endocytosis. Molecular biology of the cell 21, 2944-2952.

Aronova, S., Wedaman, K., Anderson, S., Yates, J., 3rd, and Powers, T. (2007). Probing the membrane environment of the TOR kinases reveals functional interactions between TORC1, actin, and membrane trafficking in Saccharomyces cerevisiae. Molecular biology of the cell 18, 2779-2794.

Avruch, J., Long, X., Ortiz-Vega, S., Rapley, J., Papageorgiou, A., and Dai, N. (2009). Amino acid regulation of TOR complex 1. American journal of physiology Endocrinology and metabolism 296, E592-602.

Bach, A.S., Enjalbert, S., Comunale, F., Bodin, S., Vitale, N., Charrasse, S., and Gauthier-Rouviere, C. (2010). ADP-ribosylation factor 6 regulates mammalian myoblast fusion through phospholipase D1 and phosphatidylinositol 4,5-bisphosphate signaling pathways. Molecular biology of the cell 21, 2412-2424.

Backer, J.M. (2008). The regulation and function of Class III PI3Ks: novel roles for Vps34. The Biochemical journal 410, 1-17.

Bae, C.D., Min, D.S., Fleming, I.N., and Exton, J.H. (1998). Determination of interaction sites on the small G protein RhoA for phospholipase D. The Journal of biological chemistry 273, 11596-11604.

Bai, X., Ma, D., Liu, A., Shen, X., Wang, Q.J., Liu, Y., and Jiang, Y. (2007). Rheb activates mTOR by antagonizing its endogenous inhibitor, FKBP38. Science 318, 977-980.

Banno, Y., Takuwa, Y., Yamada, M., Takuwa, N., Ohguchi, K., Hara, A., and Nozawa, Y. (2003). Involvement of phospholipase D in insulin-like growth factor-I-induced activation of extracellular signal-regulated kinase, but not phosphoinositide 3-kinase or Akt, in Chinese hamster ovary cells. The Biochemical journal 369, 363-368.

Bernardi, R., Guernah, I., Jin, D., Grisendi, S., Alimonti, A., Teruya-Feldstein, J., Cordon-Cardo, C., Simon, M.C., Rafii, S., and Pandolfi, P.P. (2006). PML inhibits HIF-1alpha translation and neoangiogenesis through repression of mTOR. Nature 442, 779-785.

Bodemann, B.O., and White, M.A. (2008). Ral GTPases and cancer: linchpin support of the tumorigenic platform. Nature reviews Cancer 8, 133-140.

Bordone, L., and Guarente, L. (2005). Calorie restriction, SIRT1 and metabolism: understanding longevity. Nature reviews Molecular cell biology 6, 298-305.

Boulay, P.L., Cotton, M., Melancon, P., and Claing, A. (2008). ADP-ribosylation factor 1 controls the activation of the phosphatidylinositol 3-kinase pathway to regulate epidermal growth factor-dependent growth and migration of breast cancer cells. The Journal of biological chemistry 283, 36425-36434.

Boulay, P.L., Schlienger, S., Lewis-Saravalli, S., Vitale, N., Ferbeyre, G., and Claing, A. (2011). ARF1 controls proliferation of breast cancer cells by regulating the retinoblastoma protein. Oncogene.

Brown, F.D., Thompson, N., Saqib, K.M., Clark, J.M., Powner, D., Thompson, N.T., Solari, R., and Wakelam, M.J. (1998). Phospholipase D1 localises to secretory granules and lysosomes and is plasma-membrane translocated on cellular stimulation. Current biology : CB 8, 835-838.

Brown, H.A., Gutowski, S., Moomaw, C.R., Slaughter, C., and Sternweis, P.C. (1993). ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity. Cell 75, 1137-1144.

Bryk, B., Hahn, K., Cohen, S.M., and Teleman, A.A. (2010). MAP4K3 regulates body size and metabolism in Drosophila. Developmental biology 344, 150-157.

Byfield, M.P., Murray, J.T., and Backer, J.M. (2005). hVps34 is a nutrient-regulated lipid kinase required for activation of p70 S6 kinase. The Journal of biological chemistry 280, 33076-33082.

Camonis, J.H., and White, M.A. (2005). Ral GTPases: corrupting the exocyst in cancer cells. Trends in cell biology 15, 327-332.

Cardenas, M.E., and Heitman, J. (1995). FKBP12-rapamycin target TOR2 is a vacuolar protein with an associated phosphatidylinositol-4 kinase activity. The EMBO journal 14, 5892-5907.

Cavenagh, M.M., Whitney, J.A., Carroll, K., Zhang, C., Boman, A.L., Rosenwald, A.G., Mellman, I., and Kahn, R.A. (1996). Intracellular distribution of Arf proteins in mammalian cells. Arf6 is uniquely localized to the plasma membrane. The Journal of biological chemistry 271, 21767-21774.

Chae, Y.C., Kim, J.H., Kim, K.L., Kim, H.W., Lee, H.Y., Heo, W.D., Meyer, T., Suh, P.G., and Ryu, S.H. (2008). Phospholipase D activity regulates integrin-mediated cell spreading and migration by inducing GTP-Rac translocation to the plasma membrane. Molecular biology of the cell 19, 3111-3123.

Chardin, P., and Tavitian, A. (1986). The ral gene: a new ras related gene isolated by the use of a synthetic probe. The EMBO journal 5, 2203-2208.

Chen, Y., Zheng, Y., and Foster, D.A. (2003). Phospholipase D confers rapamycin resistance in human breast cancer cells. Oncogene 22, 3937-3942.

Chen, Y.G., Wang, Z., Ma, J., Zhang, L., and Lu, Z. (2007). Endofin, a FYVE domain protein, interacts with Smad4 and facilitates transforming growth factor-beta signaling. The Journal of biological chemistry 282, 9688-9695.

Chien, Y., Kim, S., Bumeister, R., Loo, Y.M., Kwon, S.W., Johnson, C.L., Balakireva, M.G., Romeo, Y., Kopelovich, L., Gale, M., Jr., et al. (2006). RalB GTPase-mediated activation of the IkappaB family kinase TBK1 couples innate immune signaling to tumor cell survival. Cell 127, 157-170.

Choi, S.Y., Huang, P., Jenkins, G.M., Chan, D.C., Schiller, J., and Frohman, M.A. (2006). A common lipid links Mfn-mediated mitochondrial fusion and SNARE-regulated exocytosis. Nature cell biology 8, 1255-1262.

Cockcroft, S., Thomas, G.M., Fensome, A., Geny, B., Cunningham, E., Gout, I., Hiles, I., Totty, N.F., Truong, O., and Hsuan, J.J. (1994). Phospholipase D: a downstream effector of ARF in granulocytes. Science 263, 523-526.

Colicelli, J. (2004). Human RAS superfamily proteins and related GTPases. Science's STKE : signal transduction knowledge environment 2004, RE13.

Colley, W.C., Sung, T.C., Roll, R., Jenco, J., Hammond, S.M., Altshuller, Y., Bar-Sagi, D., Morris, A.J., and Frohman, M.A. (1997). Phospholipase D2, a distinct phospholipase D isoform with novel regulatory properties that provokes cytoskeletal reorganization. Current biology : CB 7, 191-201.

Corradetti, M.N., and Guan, K.L. (2006). Upstream of the mammalian target of rapamycin: do all roads pass through mTOR? Oncogene 25, 6347-6360.

Corradetti, M.N., Inoki, K., Bardeesy, N., DePinho, R.A., and Guan, K.L. (2004). Regulation of the TSC pathway by LKB1: evidence of a molecular link between tuberous sclerosis complex and Peutz-Jeghers syndrome. Genes & development 18, 1533-1538.

Corrotte, M., Nyguyen, A.P., Harlay, M.L., Vitale, N., Bader, M.F., and Grant, N.J. (2010). Ral isoforms are implicated in Fc gamma R-mediated phagocytosis: activation of phospholipase D by RalA. J Immunol 185, 2942-2950.

Corvera, S. (2000). Signal transduction: stuck with FYVE domains. Science's STKE : signal transduction knowledge environment 2000, pe1.

Dana, R.R., Eigsti, C., Holmes, K.L., and Leto, T.L. (2000). A regulatory role for ADP-ribosylation factor 6 (ARF6) in activation of the phagocyte NADPH oxidase. The Journal of biological chemistry 275, 32566-32571.

Dann, S.G., and Thomas, G. (2006). The amino acid sensitive TOR pathway from yeast to mammals. FEBS letters 580, 2821-2829.

Del Pozo, M.A., Kiosses, W.B., Alderson, N.B., Meller, N., Hahn, K.M., and Schwartz, M.A. (2002). Integrins regulate GTP-Rac localized effector interactions through dissociation of Rho-GDI. Nature cell biology 4, 232-239.

DerMardirossian, C., and Bokoch, G.M. (2005). GDIs: central regulatory molecules in Rho GTPase activation. Trends in cell biology 15, 356-363.

DerMardirossian, C., Schnelzer, A., and Bokoch, G.M. (2004). Phosphorylation of RhoGDI by Pak1 mediates dissociation of Rac GTPase. Molecular cell 15, 117-127.

Di Fulvio, M., and Gomez-Cambronero, J. (2005). Phospholipase D (PLD) gene expression in human neutrophils and HL-60 differentiation. Journal of leukocyte biology 77, 999-1007.

Di Paolo, G., and De Camilli, P. (2006). Phosphoinositides in cell regulation and membrane dynamics. Nature 443, 651-657.

Donaldson, J.G. (2003). Multiple roles for Arf6: sorting, structuring, and signaling at the plasma membrane. The Journal of biological chemistry 278, 41573-41576.

Donaldson, J.G., and Honda, A. (2005). Localization and function of Arf family GTPases. Biochemical Society transactions 33, 639-642.

Donaldson, J.G., and Jackson, C.L. (2011). ARF family G proteins and their regulators: roles in membrane transport, development and disease. Nature reviews Molecular cell biology 12, 362-375.

Downward, J. (2003). Targeting RAS signalling pathways in cancer therapy. Nature reviews Cancer 3, 11-22.

D'Souza-Schorey, C., and Chavrier, P. (2006). ARF proteins: roles in membrane traffic and beyond. Nature reviews Molecular cell biology 7, 347-358.

Du, G., Altshuller, Y.M., Vitale, N., Huang, P., Chasserot-Golaz, S., Morris, A.J., Bader, M.F., and Frohman, M.A. (2003). Regulation of phospholipase D1 subcellular cycling through coordination of multiple membrane association motifs. The Journal of cell biology 162, 305-315.

Efeyan, A., and Sabatini, D.M. (2010). mTOR and cancer: many loops in one pathway. Current opinion in cell biology 22, 169-176.

Eldar, H., Ben-Av, P., Schmidt, U.S., Livneh, E., and Liscovitch, M. (1993). Up-regulation of phospholipase D activity induced by overexpression of protein kinase C-alpha. Studies in intact Swiss/3T3 cells and in detergent-solubilized membranes in vitro. The Journal of biological chemistry 268, 12560-12564.

Ellson, C.D., Andrews, S., Stephens, L.R., and Hawkins, P.T. (2002). The PX domain: a new phosphoinositide-binding module. Journal of cell science 115, 1099-1105.

Engelman, J.A., Luo, J., and Cantley, L.C. (2006). The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nature reviews Genetics 7, 606-619.

Exton, J.H. (2002). Regulation of phospholipase D. FEBS letters 531, 58-61.

Falsetti, S.C., Wang, D.A., Peng, H., Carrico, D., Cox, A.D., Der, C.J., Hamilton, A.D., and Sebti, S.M. (2007). Geranylgeranyltransferase I inhibitors target RalB to inhibit anchorage-dependent growth and induce apoptosis and RalA to inhibit anchorage-independent

growth. Molecular and cellular biology 27, 8003-8014.

Fang, Y., Park, I.H., Wu, A.L., Du, G., Huang, P., Frohman, M.A., Walker, S.J., Brown, H.A., and Chen, J. (2003). PLD1 regulates mTOR signaling and mediates Cdc42 activation of S6K1. Current biology : CB 13, 2037-2044.

Fang, Y., Vilella-Bach, M., Bachmann, R., Flanigan, A., and Chen, J. (2001). Phosphatidic acid-mediated mitogenic activation of mTOR signaling. Science 294, 1942-1945.

Feig, L.A. (2003). Ral-GTPases: approaching their 15 minutes of fame. Trends in cell biology 13, 419-425.

Fingar, D.C., and Blenis, J. (2004). Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. Oncogene 23, 3151-3171.

Fingar, D.C., Richardson, C.J., Tee, A.R., Cheatham, L., Tsou, C., and Blenis, J. (2004). mTOR controls cell cycle progression through its cell growth effectors S6K1 and 4E-BP1/eukaryotic translation initiation factor 4E. Molecular and cellular biology 24, 200-216.

Foster, D.A. (2007). Regulation of mTOR by phosphatidic acid? Cancer research 67, 1-4.

Foster, D.A. (2009). Phosphatidic acid signaling to mTOR: signals for the survival of human cancer cells. Biochimica et biophysica acta 1791, 949-955.

Foster, D.A., and Xu, L. (2003). Phospholipase D in cell proliferation and cancer. Molecular cancer research : MCR 1, 789-800.

Foster, K.G., and Fingar, D.C. (2010). Mammalian target of rapamycin (mTOR): conducting the cellular signaling symphony. The Journal of biological chemistry 285, 14071-14077.

Freyberg, Z., Sweeney, D., Siddhanta, A., Bourgoin, S., Frohman, M., and Shields, D. (2001). Intracellular localization of phospholipase D1 in mammalian cells. Molecular biology of the cell 12, 943-955.

Frohman, M.A., Sung, T.C., and Morris, A.J. (1999). Mammalian phospholipase D structure and regulation. Biochimica et biophysica acta 1439, 175-186.

Frondorf, K., Henkels, K.M., Frohman, M.A., and Gomez-Cambronero, J. (2010). Phosphatidic acid is a leukocyte chemoattractant that acts through S6 kinase signaling. The Journal of biological chemistry 285, 15837-15847.

Furuya, T., Kim, M., Lipinski, M., Li, J., Kim, D., Lu, T., Shen, Y., Rameh, L., Yankner, B., Tsai, L.H., et al. (2010). Negative regulation of Vps34 by Cdk mediated phosphorylation. Molecular cell 38, 500-511.

Gadir, N., Lee, E., Garcia, A., Toschi, A., and Foster, D.A. (2007). Suppression of TGF-beta signaling by phospholipase D. Cell Cycle 6, 2840-2845.

Gao, N., Flynn, D.C., Zhang, Z., Zhong, X.S., Walker, V., Liu, K.J., Shi, X., and Jiang, B.H. (2004). G1 cell cycle progression and the expression of G1 cyclins are regulated by PI3K/AKT/mTOR/p70S6K1 signaling in human ovarian cancer cells. American journal of physiology Cell physiology 287, C281-291.

Garcia, A., Zheng, Y., Zhao, C., Toschi, A., Fan, J., Shraibman, N., Brown, H.A., Bar-Sagi, D., Foster, D.A., and Arbiser, J.L. (2008). Honokiol suppresses survival signals mediated by Ras-dependent phospholipase D activity in human cancer cells. Clinical cancer research : an official journal of the American Association for Cancer Research 14, 4267-4274.

Garcia-Martinez, J.M., and Alessi, D.R. (2008). mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1). The Biochemical journal 416, 375-385.

Gaullier, J.M., Simonsen, A., D'Arrigo, A., Bremnes, B., Stenmark, H., and Aasland, R. (1998). FYVE fingers bind PtdIns(3)P. Nature 394, 432-433.

Gingras, A.C., Raught, B., and Sonenberg, N. (2001). Regulation of translation initiation by FRAP/mTOR. Genes & development 15, 807-826.

Goberdhan, D.C. (2010). Intracellular amino acid sensing and mTORC1-regulated growth: new ways to block an old target? Curr Opin Investig Drugs 11, 1360-1367.

Gomez-Cambronero, J. (2010). New concepts in phospholipase D signaling in inflammation and cancer. TheScientificWorldJournal 10, 1356-1369.

Grant, B.D., and Donaldson, J.G. (2009). Pathways and mechanisms of endocytic recycling. Nature reviews Molecular cell biology 10, 597-608.

Guba, M., von Breitenbuch, P., Steinbauer, M., Koehl, G., Flegel, S., Hornung, M., Bruns, C.J., Zuelke, C., Farkas, S., Anthuber, M., et al. (2002). Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. Nature medicine 8, 128-135.

Guertin, D.A., and Sabatini, D.M. (2007). Defining the role of mTOR in cancer. Cancer cell 12, 9-22.

Guertin, D.A., Stevens, D.M., Thoreen, C.C., Burds, A.A., Kalaany, N.Y., Moffat, J., Brown, M., Fitzgerald, K.J., and Sabatini, D.M. (2006). Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1. Developmental cell 11, 859-871.

Gulati, P., Gaspers, L.D., Dann, S.G., Joaquin, M., Nobukuni, T., Natt, F., Kozma, S.C., Thomas, A.P., and Thomas, G. (2008). Amino acids activate mTOR complex 1 via Ca2+/CaM signaling to hVps34. Cell metabolism 7, 456-465.

Gulati, P., and Thomas, G. (2007). Nutrient sensing in the mTOR/S6K1 signalling pathway. Biochemical Society transactions 35, 236-238.

Gwinn, D.M., Shackelford, D.B., Egan, D.F., Mihaylova, M.M., Mery, A., Vasquez, D.S., Turk, B.E., and Shaw, R.J. (2008). AMPK phosphorylation of raptor mediates a metabolic checkpoint. Molecular cell 30, 214-226.

Ha, S.H., Kim, D.H., Kim, I.S., Kim, J.H., Lee, M.N., Lee, H.J., Jang, S.K., Suh, P.G., and Ryu, S.H. (2006). PLD2 forms a functional complex with mTOR/raptor to transduce mitogenic signals. Cellular signalling 18, 2283-2291.

Hammond, S.M., Altshuller, Y.M., Sung, T.C., Rudge, S.A., Rose, K., Engebrecht, J., Morris, A.J., and Frohman, M.A. (1995). Human ADP-ribosylation factor-activated phosphatidylcholine-specific phospholipase D defines a new and highly conserved gene family. The Journal of biological chemistry 270, 29640-29643.

Hammond, S.M., Jenco, J.M., Nakashima, S., Cadwallader, K., Gu, Q., Cook, S., Nozawa, Y., Prestwich, G.D., Frohman, M.A., and Morris, A.J. (1997). Characterization of two alternately spliced forms of phospholipase D1. Activation of the purified enzymes by phosphatidylinositol 4,5-bisphosphate, ADP-ribosylation factor, and Rho family monomeric GTP-binding proteins and protein kinase C-alpha. The Journal of biological chemistry 272, 3860-3868.

Hara, K., Maruki, Y., Long, X., Yoshino, K., Oshiro, N., Hidayat, S., Tokunaga, C., Avruch, J., and Yonezawa, K. (2002). Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell 110, 177-189.

Harris, T.E., and Lawrence, J.C., Jr. (2003). TOR signaling. Science's STKE : signal transduction knowledge environment 2003, re15.

Hashimoto, S., Onodera, Y., Hashimoto, A., Tanaka, M., Hamaguchi, M., Yamada, A., and Sabe, H. (2004). Requirement for Arf6 in breast cancer invasive activities. Proceedings of the National Academy of Sciences of the United States of America 101, 6647-6652.

Hayakawa, A., Hayes, S., Leonard, D., Lambright, D., and Corvera, S. (2007). Evolutionarily conserved structural and functional roles of the FYVE domain. Biochemical Society symposium, 95-105.

Herman, P.K., Stack, J.H., DeModena, J.A., and Emr, S.D. (1991). A novel protein kinase homolog essential for protein sorting to the yeast lysosome-like vacuole. Cell 64, 425-437.

Hiroaki, H., Ago, T., Ito, T., Sumimoto, H., and Kohda, D. (2001). Solution structure of the PX domain, a target of the SH3 domain. Nature structural biology 8, 526-530.

Hiroyama, M., and Exton, J.H. (2005). Localization and regulation of phospholipase D2 by ARF6. Journal of cellular biochemistry 95, 149-164.

Hitomi, T., Zhang, J., Nicoletti, L.M., Grodzki, A.C., Jamur, M.C., Oliver, C., and Siraganian, R.P. (2004). Phospholipase D1 regulates high-affinity IgE receptor-induced mast cell degranulation. Blood 104, 4122-4128.

Hodgkin, M.N., Masson, M.R., Powner, D., Saqib, K.M., Ponting, C.P., and Wakelam, M.J.

(2000). Phospholipase D regulation and localisation is dependent upon a phosphatidylinositol 4,5-biphosphate-specific PH domain. Current biology : CB 10, 43-46.

Honda, A., Al-Awar, O.S., Hay, J.C., and Donaldson, J.G. (2005). Targeting of Arf-1 to the early Golgi by membrin, an ER-Golgi SNARE. The Journal of cell biology 168, 1039-1051.

Hornberger, T.A., Chu, W.K., Mak, Y.W., Hsiung, J.W., Huang, S.A., and Chien, S. (2006). The role of phospholipase D and phosphatidic acid in the mechanical activation of mTOR signaling in skeletal muscle. Proceedings of the National Academy of Sciences of the United States of America 103, 4741-4746.

Hornia, A., Lu, Z., Sukezane, T., Zhong, M., Joseph, T., Frankel, P., and Foster, D.A. (1999). Antagonistic effects of protein kinase C alpha and delta on both transformation and phospholipase D activity mediated by the epidermal growth factor receptor. Molecular and cellular biology 19, 7672-7680.

Hu, T., and Exton, J.H. (2003). Mechanisms of regulation of phospholipase D1 by protein kinase Calpha. The Journal of biological chemistry 278, 2348-2355.

Huang, P., and Frohman, M.A. (2007). The potential for phospholipase D as a new therapeutic target. Expert opinion on therapeutic targets 11, 707-716.

Hughes, W.E., Elgundi, Z., Huang, P., Frohman, M.A., and Biden, T.J. (2004). Phospholipase D1 regulates secretagogue-stimulated insulin release in pancreatic beta-cells. The Journal of biological chemistry 279, 27534-27541.

Hughes, W.E., and Parker, P.J. (2001). Endosomal localization of phospholipase D 1a and 1b is defined by the C-termini of the proteins, and is independent of activity. The Biochemical journal 356, 727-736.

Ikenoue, T., Inoki, K., Yang, Q., Zhou, X., and Guan, K.L. (2008). Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling. The EMBO journal 27, 1919-1931.

Inoki, K., Li, Y., Xu, T., and Guan, K.L. (2003). Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes & development 17, 1829-1834.

Inoki, K., Li, Y., Zhu, T., Wu, J., and Guan, K.L. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nature cell biology 4, 648-657.

Inoki, K., Ouyang, H., Zhu, T., Lindvall, C., Wang, Y., Zhang, X., Yang, Q., Bennett, C., Harada, Y., Stankunas, K., et al. (2006). TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth. Cell 126, 955-968.

Inoki, K., Zhu, T., and Guan, K.L. (2003). TSC2 mediates cellular energy response to control cell growth and survival. Cell 115, 577-590.

Iyer, S.S., Barton, J.A., Bourgoin, S., and Kusner, D.J. (2004). Phospholipases D1 and D2

coordinately regulate macrophage phagocytosis. J Immunol 173, 2615-2623.

Jacinto, E., Facchinetti, V., Liu, D., Soto, N., Wei, S., Jung, S.Y., Huang, Q., Qin, J., and Su, B. (2006). SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. Cell 127, 125-137.

Jacinto, E., and Hall, M.N. (2003). Tor signalling in bugs, brain and brawn. Nature reviews Molecular cell biology 4, 117-126.

Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Ruegg, M.A., Hall, A., and Hall, M.N. (2004). Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nature cell biology 6, 1122-1128.

Jaffe, A.B., and Hall, A. (2002). Rho GTPases in transformation and metastasis. Advances in cancer research 84, 57-80.

Jenkins, G.M., and Frohman, M.A. (2005). Phospholipase D: a lipid centric review. Cellular and molecular life sciences : CMLS 62, 2305-2316.

Jiang, H., Luo, J.Q., Urano, T., Frankel, P., Lu, Z., Foster, D.A., and Feig, L.A. (1995). Involvement of Ral GTPase in v-Src-induced phospholipase D activation. Nature 378, 409-412.

Jones, A.C., Shyamsundar, M.M., Thomas, M.W., Maynard, J., Idziaszczyk, S., Tomkins, S., Sampson, J.R., and Cheadle, J.P. (1999). Comprehensive mutation analysis of TSC1 and TSC2-and phenotypic correlations in 150 families with tuberous sclerosis. American journal of human genetics 64, 1305-1315.

Joseph, T., Bryant, A., Frankel, P., Wooden, R., Kerkhoff, E., Rapp, U.R., and Foster, D.A. (2002). Phospholipase D overcomes cell cycle arrest induced by high-intensity Raf signaling. Oncogene 21, 3651-3658.

Jovanovic, O.A., Brown, F.D., and Donaldson, J.G. (2006). An effector domain mutant of Arf6 implicates phospholipase D in endosomal membrane recycling. Molecular biology of the cell 17, 327-335.

Juhasz, G., Hill, J.H., Yan, Y., Sass, M., Baehrecke, E.H., Backer, J.M., and Neufeld, T.P. (2008). The class III PI(3)K Vps34 promotes autophagy and endocytosis but not TOR signaling in Drosophila. The Journal of cell biology 181, 655-666.

Jung, E.M., Betancourt-Calle, S., Mann-Blakeney, R., Griner, R.D., and Bollinger Bollag, W. (1999). Sustained phospholipase D activation is associated with keratinocyte differentiation. Carcinogenesis 20, 569-576.

Kanaho, Y., Funakoshi, Y., and Hasegawa, H. (2009). Phospholipase D signalling and its involvement in neurite outgrowth. Biochimica et biophysica acta 1791, 898-904.

Kang, D.W., Lee, S.H., Yoon, J.W., Park, W.S., Choi, K.Y., and Min do, S. (2010). Phospholipase D1 drives a positive feedback loop to reinforce the Wnt/beta-catenin/TCF

signaling axis. Cancer research 70, 4233-4242.

Kapahi, P., Chen, D., Rogers, A.N., Katewa, S.D., Li, P.W., Thomas, E.L., and Kockel, L. (2010). With TOR, less is more: a key role for the conserved nutrient-sensing TOR pathway in aging. Cell metabolism 11, 453-465.

Katewa, S.D., and Kapahi, P. (2010). Dietary restriction and aging, 2009. Aging cell 9, 105-112.

Kim, D.H., Sarbassov, D.D., Ali, S.M., King, J.E., Latek, R.R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D.M. (2002). mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell 110, 163-175.

Kim, E., Goraksha-Hicks, P., Li, L., Neufeld, T.P., and Guan, K.L. (2008). Regulation of TORC1 by Rag GTPases in nutrient response. Nature cell biology 10, 935-945.

Kim, E., and Guan, K.L. (2009). RAG GTPases in nutrient-mediated TOR signaling pathway. Cell Cycle 8, 1014-1018.

Kinbara, K., Goldfinger, L.E., Hansen, M., Chou, F.L., and Ginsberg, M.H. (2003). Ras GTPases: integrins' friends or foes? Nature reviews Molecular cell biology 4, 767-776.

Klionsky, D.J. (2007). Autophagy: from phenomenology to molecular understanding in less than a decade. Nature reviews Molecular cell biology 8, 931-937.

Knoepp, S.M., Chahal, M.S., Xie, Y., Zhang, Z., Brauner, D.J., Hallman, M.A., Robinson, S.A., Han, S., Imai, M., Tomlinson, S., et al. (2008). Effects of active and inactive phospholipase D2 on signal transduction, adhesion, migration, invasion, and metastasis in EL4 lymphoma cells. Molecular pharmacology 74, 574-584.

Koharudin, L.M., Furey, W., Liu, H., Liu, Y.J., and Gronenborn, A.M. (2009). The phox domain of sorting nexin 5 lacks phosphatidylinositol 3-phosphate (PtdIns(3)P) specificity and preferentially binds to phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2). The Journal of biological chemistry 284, 23697-23707.

Kunz, J., Schneider, U., Howald, I., Schmidt, A., and Hall, M.N. (2000). HEAT repeats mediate plasma membrane localization of Tor2p in yeast. The Journal of biological chemistry 275, 37011-37020.

Kwiatkowski, D.J., and Manning, B.D. (2005). Tuberous sclerosis: a GAP at the crossroads of multiple signaling pathways. Human molecular genetics 14 Spec No. 2, R251-258.

Kwun, H.J., Lee, J.H., Min, D.S., and Jang, K.L. (2003). Transcriptional repression of cyclin-dependent kinase inhibitor p21 gene by phospholipase D1 and D2. FEBS letters 544, 38-44.

Lalli, G., and Hall, A. (2005). Ral GTPases regulate neurite branching through GAP-43 and the exocyst complex. The Journal of cell biology 171, 857-869.

Law, M., Forrester, E., Chytil, A., Corsino, P., Green, G., Davis, B., Rowe, T., and Law, B. (2006). Rapamycin disrupts cyclin/cyclin-dependent kinase/p21/proliferating cell nuclear antigen complexes and cyclin D1 reverses rapamycin action by stabilizing these complexes. Cancer research 66, 1070-1080.

Lawe, D.C., Patki, V., Heller-Harrison, R., Lambright, D., and Corvera, S. (2000). The FYVE domain of early endosome antigen 1 is required for both phosphatidylinositol 3-phosphate and Rab5 binding. Critical role of this dual interaction for endosomal localization. The Journal of biological chemistry 275, 3699-3705.

Lee, C.S., Kim, I.S., Park, J.B., Lee, M.N., Lee, H.Y., Suh, P.G., and Ryu, S.H. (2006). The phox homology domain of phospholipase D activates dynamin GTPase activity and accelerates EGFR endocytosis. Nature cell biology 8, 477-484.

Lee, C.S., Kim, K.L., Jang, J.H., Choi, Y.S., Suh, P.G., and Ryu, S.H. (2009). The roles of phospholipase D in EGFR signaling. Biochimica et biophysica acta 1791, 862-868.

Lee, S.A., Kovacs, J., Stahelin, R.V., Cheever, M.L., Overduin, M., Setty, T.G., Burd, C.G., Cho, W., and Kutateladze, T.G. (2006). Molecular mechanism of membrane docking by the Vam7p PX domain. The Journal of biological chemistry 281, 37091-37101.

Lehman, N., Ledford, B., Di Fulvio, M., Frondorf, K., McPhail, L.C., and Gomez-Cambronero, J. (2007). Phospholipase D2-derived phosphatidic acid binds to and activates ribosomal p70 S6 kinase independently of mTOR. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology 21, 1075-1087.

Lemmon, M.A. (2003). Phosphoinositide recognition domains. Traffic 4, 201-213.

Levine, B., and Klionsky, D.J. (2004). Development by self-digestion: molecular mechanisms and biological functions of autophagy. Developmental cell 6, 463-477.

Li, H., Tsang, C.K., Watkins, M., Bertram, P.G., and Zheng, X.F. (2006). Nutrient regulates Tor1 nuclear localization and association with rDNA promoter. Nature 442, 1058-1061.

Lim, K.H., Baines, A.T., Fiordalisi, J.J., Shipitsin, M., Feig, L.A., Cox, A.D., Der, C.J., and Counter, C.M. (2005). Activation of RalA is critical for Ras-induced tumorigenesis of human cells. Cancer cell 7, 533-545.

Lim, K.H., Brady, D.C., Kashatus, D.F., Ancrile, B.B., Der, C.J., Cox, A.D., and Counter, C.M. (2010). Aurora-A phosphorylates, activates, and relocalizes the small GTPase RalA. Molecular and cellular biology 30, 508-523.

Liu, L., Liao, H., Castle, A., Zhang, J., Casanova, J., Szabo, G., and Castle, D. (2005). SCAMP2 interacts with Arf6 and phospholipase D1 and links their function to exocytotic fusion pore formation in PC12 cells. Molecular biology of the cell 16, 4463-4472.

Loewith, R., Jacinto, E., Wullschleger, S., Lorberg, A., Crespo, J.L., Bonenfant, D., Oppliger, W., Jenoe, P., and Hall, M.N. (2002). Two TOR complexes, only one of which is rapamycin

sensitive, have distinct roles in cell growth control. Molecular cell 10, 457-468.

Long, X., Ortiz-Vega, S., Lin, Y., and Avruch, J. (2005). Rheb binding to mammalian target of rapamycin (mTOR) is regulated by amino acid sufficiency. The Journal of biological chemistry 280, 23433-23436.

Luo, J.Q., Liu, X., Frankel, P., Rotunda, T., Ramos, M., Flom, J., Jiang, H., Feig, L.A., Morris, A.J., Kahn, R.A., et al. (1998). Functional association between Arf and RalA in active phospholipase D complex. Proceedings of the National Academy of Sciences of the United States of America 95, 3632-3637.

Luo, J.Q., Liu, X., Hammond, S.M., Colley, W.C., Feig, L.A., Frohman, M.A., Morris, A.J., and Foster, D.A. (1997). RalA interacts directly with the Arf-responsive, PIP2-dependent phospholipase D1. Biochemical and biophysical research communications 235, 854-859.

Ma, D., Bai, X., Guo, S., and Jiang, Y. (2008). The switch I region of Rheb is critical for its interaction with FKBP38. The Journal of biological chemistry 283, 25963-25970.

Ma, L., Chen, Z., Erdjument-Bromage, H., Tempst, P., and Pandolfi, P.P. (2005). Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis. Cell 121, 179-193.

Ma, W.N., Park, S.Y., and Han, J.S. (2010). Role of phospholipase D1 in glucose-induced insulin secretion in pancreatic Beta cells. Experimental & molecular medicine 42, 456-464.

Ma, X.M., and Blenis, J. (2009). Molecular mechanisms of mTOR-mediated translational control. Nature reviews Molecular cell biology 10, 307-318.

Maehama, T., Tanaka, M., Nishina, H., Murakami, M., Kanaho, Y., and Hanada, K. (2008). RalA functions as an indispensable signal mediator for the nutrient-sensing system. The Journal of biological chemistry 283, 35053-35059.

Malliri, A., and Collard, J.G. (2003). Role of Rho-family proteins in cell adhesion and cancer. Current opinion in cell biology 15, 583-589.

Manning, B.D., Tee, A.R., Logsdon, M.N., Blenis, J., and Cantley, L.C. (2002). Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. Molecular cell 10, 151-162.

Mari, M., and Reggiori, F. (2007). Shaping membranes into autophagosomes. Nature cell biology 9, 1125-1127.

Martin, T.D., Samuel, J.C., Routh, E.D., Der, C.J., and Yeh, J.J. (2011). Activation and involvement of Ral GTPases in colorectal cancer. Cancer research 71, 206-215.

Massenburg, D., Han, J.S., Liyanage, M., Patton, W.A., Rhee, S.G., Moss, J., and Vaughan, M. (1994). Activation of rat brain phospholipase D by ADP-ribosylation factors 1,5, and 6: separation of ADP-ribosylation factor-dependent and oleate-dependent enzymes. Proceedings of

the National Academy of Sciences of the United States of America 91, 11718-11722.

Mathew, R., Karantza-Wadsworth, V., and White, E. (2007). Role of autophagy in cancer. Nature reviews Cancer 7, 961-967.

Melendez, A.J., Harnett, M.M., and Allen, J.M. (2001). Crosstalk between ARF6 and protein kinase Calpha in Fc(gamma)RI-mediated activation of phospholipase D1. Current biology : CB 11, 869-874.

Mitin, N., Rossman, K.L., and Der, C.J. (2005). Signaling interplay in Ras superfamily function. Current biology : CB 15, R563-574.

Mizushima, N., Levine, B., Cuervo, A.M., and Klionsky, D.J. (2008). Autophagy fights disease through cellular self-digestion. Nature 451, 1069-1075.

Mor, A., Campi, G., Du, G., Zheng, Y., Foster, D.A., Dustin, M.L., and Philips, M.R. (2007). The lymphocyte function-associated antigen-1 receptor costimulates plasma membrane Ras via phospholipase D2. Nature cell biology 9, 713-719.

Morishige, M., Hashimoto, S., Ogawa, E., Toda, Y., Kotani, H., Hirose, M., Wei, S., Hashimoto, A., Yamada, A., Yano, H., et al. (2008). GEP100 links epidermal growth factor receptor signalling to Arf6 activation to induce breast cancer invasion. Nature cell biology 10, 85-92.

Morris, A.J., Engebrecht, J., and Frohman, M.A. (1996). Structure and regulation of phospholipase D. Trends in pharmacological sciences 17, 182-185.

Moss, J., and Vaughan, M. (1998). Molecules in the ARF orbit. The Journal of biological chemistry 273, 21431-21434.

Muralidharan-Chari, V., Hoover, H., Clancy, J., Schweitzer, J., Suckow, M.A., Schroeder, V., Castellino, F.J., Schorey, J.S., and D'Souza-Schorey, C. (2009). ADP-ribosylation factor 6 regulates tumorigenic and invasive properties in vivo. Cancer research 69, 2201-2209.

Murray, J.T., Panaretou, C., Stenmark, H., Miaczynska, M., and Backer, J.M. (2002). Role of Rab5 in the recruitment of hVps34/p150 to the early endosome. Traffic 3, 416-427.

Nielsen, E., Cheung, A.Y., and Ueda, T. (2008). The regulatory RAB and ARF GTPases for vesicular trafficking. Plant physiology 147, 1516-1526.

Nishikimi, A., Fukuhara, H., Su, W., Hongu, T., Takasuga, S., Mihara, H., Cao, Q., Sanematsu, F., Kanai, M., Hasegawa, H., et al. (2009). Sequential regulation of DOCK2 dynamics by two phospholipids during neutrophil chemotaxis. Science 324, 384-387.

Nobukuni, T., Joaquin, M., Roccio, M., Dann, S.G., Kim, S.Y., Gulati, P., Byfield, M.P., Backer, J.M., Natt, F., Bos, J.L., et al. (2005). Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. Proceedings of the National Academy of Sciences of the United States of America 102, 14238-14243.

O'Luanaigh, N., Pardo, R., Fensome, A., Allen-Baume, V., Jones, D., Holt, M.R., and Cockcroft, S. (2002). Continual production of phosphatidic acid by phospholipase D is essential for antigen-stimulated membrane ruffling in cultured mast cells. Molecular biology of the cell 13, 3730-3746.

Pai, J.K., Pachter, J.A., Weinstein, I.B., and Bishop, W.R. (1991). Overexpression of protein kinase C beta 1 enhances phospholipase D activity and diacylglycerol formation in phorbol ester-stimulated rat fibroblasts. Proceedings of the National Academy of Sciences of the United States of America 88, 598-602.

Park, M.H., Ahn, B.H., Hong, Y.K., and Min do, S. (2009). Overexpression of phospholipase D enhances matrix metalloproteinase-2 expression and glioma cell invasion via protein kinase C and protein kinase A/NF-kappaB/Sp1-mediated signaling pathways. Carcinogenesis 30, 356-365.

Pearce, L.R., Huang, X., Boudeau, J., Pawlowski, R., Wullschleger, S., Deak, M., Ibrahim, A.F., Gourlay, R., Magnuson, M.A., and Alessi, D.R. (2007). Identification of Protor as a novel Rictor-binding component of mTOR complex-2. The Biochemical journal 405, 513-522.

Perez-Mansilla, B., Ha, V.L., Justin, N., Wilkins, A.J., Carpenter, C.L., and Thomas, G.M. (2006). The differential regulation of phosphatidylinositol 4-phosphate 5-kinases and phospholipase D1 by ADP-ribosylation factors 1 and 6. Biochimica et biophysica acta 1761, 1429-1442.

Perry, J., and Kleckner, N. (2003). The ATRs, ATMs, and TORs are giant HEAT repeat proteins. Cell 112, 151-155.

Peterson, T.R., Laplante, M., Thoreen, C.C., Sancak, Y., Kang, S.A., Kuehl, W.M., Gray, N.S., and Sabatini, D.M. (2009). DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. Cell 137, 873-886.

Powner, D.J., Hodgkin, M.N., and Wakelam, M.J. (2002). Antigen-stimulated activation of phospholipase D1b by Rac1, ARF6, and PKCalpha in RBL-2H3 cells. Molecular biology of the cell 13, 1252-1262.

Rangarajan, A., Hong, S.J., Gifford, A., and Weinberg, R.A. (2004). Species- and cell type-specific requirements for cellular transformation. Cancer cell 6, 171-183.

Raught, B., Gingras, A.C., Gygi, S.P., Imataka, H., Morino, S., Gradi, A., Aebersold, R., and Sonenberg, N. (2000). Serum-stimulated, rapamycin-sensitive phosphorylation sites in the eukaryotic translation initiation factor 4GI. The EMBO journal 19, 434-444.

Resnik-Docampo, M., and de Celis, J.F. (2011). MAP4K3 is a component of the TORC1 signalling complex that modulates cell growth and viability in Drosophila melanogaster. PloS one 6, e14528.

Ridley, A.J., Schwartz, M.A., Burridge, K., Firtel, R.A., Ginsberg, M.H., Borisy, G., Parsons, J.T., and Horwitz, A.R. (2003). Cell migration: integrating signals from front to back. Science 302, 1704-1709.

Roccio, M., Bos, J.L., and Zwartkruis, F.J. (2006). Regulation of the small GTPase Rheb by amino acids. Oncogene 25, 657-664.

Rudge, S.A., and Wakelam, M.J. (2009). Inter-regulatory dynamics of phospholipase D and the actin cytoskeleton. Biochimica et biophysica acta 1791, 856-861.

Russell, R.C., Fang, C., and Guan, K.L. (2011). An emerging role for TOR signaling in mammalian tissue and stem cell physiology. Development 138, 3343-3356.

Sabatini, D.M. (2006). mTOR and cancer: insights into a complex relationship. Nature reviews Cancer 6, 729-734.

Saci, A., Cantley, L.C., and Carpenter, C.L. (2011). Rac1 regulates the activity of mTORC1 and mTORC2 and controls cellular size. Molecular cell 42, 50-61.

Saito, M., Iwadate, M., Higashimoto, M., Ono, K., Takebayashi, Y., and Takenoshita, S. (2007). Expression of phospholipase D2 in human colorectal carcinoma. Oncology reports 18, 1329-1334.

Sancak, Y., Peterson, T.R., Shaul, Y.D., Lindquist, R.A., Thoreen, C.C., Bar-Peled, L., and Sabatini, D.M. (2008). The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science 320, 1496-1501.

Sancak, Y., and Sabatini, D.M. (2009). Rag proteins regulate amino-acid-induced mTORC1 signalling. Biochemical Society transactions 37, 289-290.

Sancak, Y., Thoreen, C.C., Peterson, T.R., Lindquist, R.A., Kang, S.A., Spooner, E., Carr, S.A., and Sabatini, D.M. (2007). PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. Molecular cell 25, 903-915.

Sarbassov, D.D., Ali, S.M., Kim, D.H., Guertin, D.A., Latek, R.R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D.M. (2004). Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Current biology : CB 14, 1296-1302.

Sarbassov, D.D., Ali, S.M., and Sabatini, D.M. (2005). Growing roles for the mTOR pathway. Current opinion in cell biology 17, 596-603.

Sato, T.K., Overduin, M., and Emr, S.D. (2001). Location, location, location: membrane targeting directed by PX domains. Science 294, 1881-1885.

Sawyers, C.L. (2003). Will mTOR inhibitors make it as cancer drugs? Cancer cell 4, 343-348.

Schu, P.V., Takegawa, K., Fry, M.J., Stack, J.H., Waterfield, M.D., and Emr, S.D. (1993). Phosphatidylinositol 3-kinase encoded by yeast VPS34 gene essential for protein sorting. Science 260, 88-91.

Sciorra, V.A., Rudge, S.A., Prestwich, G.D., Frohman, M.A., Engebrecht, J., and Morris, A.J.

(1999). Identification of a phosphoinositide binding motif that mediates activation of mammalian and yeast phospholipase D isoenzymes. The EMBO journal 18, 5911-5921.

Sciorra, V.A., Rudge, S.A., Wang, J., McLaughlin, S., Engebrecht, J., and Morris, A.J. (2002). Dual role for phosphoinositides in regulation of yeast and mammalian phospholipase D enzymes. The Journal of cell biology 159, 1039-1049.

Seet, L.F., and Hong, W. (2006). The Phox (PX) domain proteins and membrane traffic. Biochimica et biophysica acta 1761, 878-896.

Sengupta, S., Peterson, T.R., and Sabatini, D.M. (2010). Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. Molecular cell 40, 310-322.

Shaw, R.J. (2008). mTOR signaling: RAG GTPases transmit the amino acid signal. Trends in biochemical sciences 33, 565-568.

Shaywitz, A.J., Courtney, K.D., Patnaik, A., and Cantley, L.C. (2008). PI3K enters beta-testing. Cell metabolism 8, 179-181.

Shen, Y., Xu, L., and Foster, D.A. (2001). Role for phospholipase D in receptor-mediated endocytosis. Molecular and cellular biology 21, 595-602.

Shi, M., Zheng, Y., Garcia, A., Xu, L., and Foster, D.A. (2007). Phospholipase D provides a survival signal in human cancer cells with activated H-Ras or K-Ras. Cancer letters 258, 268-275.

Slessareva, J.E., Routt, S.M., Temple, B., Bankaitis, V.A., and Dohlman, H.G. (2006). Activation of the phosphatidylinositol 3-kinase Vps34 by a G protein alpha subunit at the endosome. Cell 126, 191-203.

Smith, E.M., Finn, S.G., Tee, A.R., Browne, G.J., and Proud, C.G. (2005). The tuberous sclerosis protein TSC2 is not required for the regulation of the mammalian target of rapamycin by amino acids and certain cellular stresses. The Journal of biological chemistry 280, 18717-18727.

Song, J., and Foster, D.A. (1993). v-Src activates a unique phospholipase D activity that can be distinguished from the phospholipase D activity activated by phorbol esters. The Biochemical journal 294 (Pt 3), 711-717.

Stack, J.H., Herman, P.K., Schu, P.V., and Emr, S.D. (1993). A membrane-associated complex containing the Vps15 protein kinase and the Vps34 PI 3-kinase is essential for protein sorting to the yeast lysosome-like vacuole. The EMBO journal 12, 2195-2204.

Stahelin, R.V., Ananthanarayanan, B., Blatner, N.R., Singh, S., Bruzik, K.S., Murray, D., and Cho, W. (2004). Mechanism of membrane binding of the phospholipase D1 PX domain. The Journal of biological chemistry 279, 54918-54926.

Stein, M.P., Feng, Y., Cooper, K.L., Welford, A.M., and Wandinger-Ness, A. (2003). Human VPS34 and p150 are Rab7 interacting partners. Traffic 4, 754-771.

Stenmark, H., Aasland, R., and Driscoll, P.C. (2002). The phosphatidylinositol 3-phosphate-binding FYVE finger. FEBS letters 513, 77-84.

Su, W., Chen, Q., and Frohman, M.A. (2009). Targeting phospholipase D with small-molecule inhibitors as a potential therapeutic approach for cancer metastasis. Future Oncol 5, 1477-1486.

Sugars, J.M., Cellek, S., Manifava, M., Coadwell, J., and Ktistakis, N.T. (2002). Hierarchy of membrane-targeting signals of phospholipase D1 involving lipid modification of a pleckstrin homology domain. The Journal of biological chemistry 277, 29152-29161.

Sun, Y., and Chen, J. (2008). mTOR signaling: PLD takes center stage. Cell Cycle 7, 3118-3123.

Sun, Y., Fang, Y., Yoon, M.S., Zhang, C., Roccio, M., Zwartkruis, F.J., Armstrong, M., Brown, H.A., and Chen, J. (2008). Phospholipase D1 is an effector of Rheb in the mTOR pathway. Proceedings of the National Academy of Sciences of the United States of America 105, 8286-8291.

Sung, T.C., Zhang, Y., Morris, A.J., and Frohman, M.A. (1999). Structural analysis of human phospholipase D1. The Journal of biological chemistry 274, 3659-3666.

Suzuki, K., and Ohsumi, Y. (2007). Molecular machinery of autophagosome formation in yeast, Saccharomyces cerevisiae. FEBS letters 581, 2156-2161.

Toda, K., Nogami, M., Murakami, K., Kanaho, Y., and Nakayama, K. (1999). Colocalization of phospholipase D1 and GTP-binding-defective mutant of ADP-ribosylation factor 6 to endosomes and lysosomes. FEBS letters 442, 221-225.

Toschi, A., Lee, E., Xu, L., Garcia, A., Gadir, N., and Foster, D.A. (2009). Regulation of mTORC1 and mTORC2 complex assembly by phosphatidic acid: competition with rapamycin. Molecular and cellular biology 29, 1411-1420.

van Dam, E.M., and Robinson, P.J. (2006). Ral: mediator of membrane trafficking. The international journal of biochemistry & cell biology 38, 1841-1847.

Vanhaesebroeck, B., Guillermet-Guibert, J., Graupera, M., and Bilanges, B. (2010). The emerging mechanisms of isoform-specific PI3K signalling. Nature reviews Molecular cell biology 11, 329-341.

Venable, M.E., and Obeid, L.M. (1999). Phospholipase D in cellular senescence. Biochimica et biophysica acta 1439, 291-298.

Veverka, V., Crabbe, T., Bird, I., Lennie, G., Muskett, F.W., Taylor, R.J., and Carr, M.D. (2008). Structural characterization of the interaction of mTOR with phosphatidic acid and a novel class of inhibitor: compelling evidence for a central role of the FRB domain in small molecule-mediated regulation of mTOR. Oncogene 27, 585-595.

Vigil, D., Cherfils, J., Rossman, K.L., and Der, C.J. (2010). Ras superfamily GEFs and GAPs: validated and tractable targets for cancer therapy? Nature reviews Cancer 10, 842-857.

Vivanco, I., and Sawyers, C.L. (2002). The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nature reviews Cancer 2, 489-501.

Volinia, S., Dhand, R., Vanhaesebroeck, B., MacDougall, L.K., Stein, R., Zvelebil, M.J., Domin, J., Panaretou, C., and Waterfield, M.D. (1995). A human phosphatidylinositol 3-kinase complex related to the yeast Vps34p-Vps15p protein sorting system. The EMBO journal 14, 3339-3348.

Wang, L., Harris, T.E., Roth, R.A., and Lawrence, J.C., Jr. (2007). PRAS40 regulates mTORC1 kinase activity by functioning as a direct inhibitor of substrate binding. The Journal of biological chemistry 282, 20036-20044.

Ward, Y., Wang, W., Woodhouse, E., Linnoila, I., Liotta, L., and Kelly, K. (2001). Signal pathways which promote invasion and metastasis: critical and distinct contributions of extracellular signal-regulated kinase and Ral-specific guanine exchange factor pathways. Molecular and cellular biology 21, 5958-5969.

Wiget, P., Shimada, Y., Butty, A.C., Bi, E., and Peter, M. (2004). Site-specific regulation of the GEF Cdc24p by the scaffold protein Far1p during yeast mating. The EMBO journal 23, 1063-1074.

Windmiller, D.A., and Backer, J.M. (2003). Distinct phosphoinositide 3-kinases mediate mast cell degranulation in response to G-protein-coupled versus FcepsilonRI receptors. The Journal of biological chemistry 278, 11874-11878.

Wishart, M.J., Taylor, G.S., and Dixon, J.E. (2001). Phoxy lipids: revealing PX domains as phosphoinositide binding modules. Cell 105, 817-820.

Woo, S.Y., Kim, D.H., Jun, C.B., Kim, Y.M., Haar, E.V., Lee, S.I., Hegg, J.W., Bandhakavi, S., and Griffin, T.J. (2007). PRR5, a novel component of mTOR complex 2, regulates platelet-derived growth factor receptor beta expression and signaling. The Journal of biological chemistry 282, 25604-25612.

Worthylake, D.K., Rossman, K.L., and Sondek, J. (2000). Crystal structure of Rac1 in complex with the guanine nucleotide exchange region of Tiam1. Nature 408, 682-688.

Wullschleger, S., Loewith, R., and Hall, M.N. (2006). TOR signaling in growth and metabolism. Cell 124, 471-484.

Xie, Z., Ho, W.T., and Exton, J.H. (1998). Association of N- and C-terminal domains of phospholipase D is required for catalytic activity. The Journal of biological chemistry 273, 34679-34682.

Xu, L., Frankel, P., Jackson, D., Rotunda, T., Boshans, R.L., D'Souza-Schorey, C., and Foster, D.A. (2003). Elevated phospholipase D activity in H-Ras- but not K-Ras-transformed cells by the synergistic action of RalA and ARF6. Molecular and cellular biology 23, 645-654.

Xu, Y., Seet, L.F., Hanson, B., and Hong, W. (2001). The Phox homology (PX) domain, a new player in phosphoinositide signalling. The Biochemical journal 360, 513-530.

Yan, L., Mieulet, V., Burgess, D., Findlay, G.M., Sully, K., Procter, J., Goris, J., Janssens, V., Morrice, N.A., and Lamb, R.F. (2010). PP2A T61 epsilon is an inhibitor of MAP4K3 in nutrient signaling to mTOR. Molecular cell 37, 633-642.

Yan, Y., Flinn, R.J., Wu, H., Schnur, R.S., and Backer, J.M. (2009). hVps15, but not Ca2+/CaM, is required for the activity and regulation of hVps34 in mammalian cells. The Biochemical journal 417, 747-755.

Yang, Q., and Guan, K.L. (2007). Expanding mTOR signaling. Cell research 17, 666-681.

Yang, Q., Inoki, K., Ikenoue, T., and Guan, K.L. (2006). Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity. Genes & development 20, 2820-2832.

Yeo, E.J., and Exton, J.H. (1995). Stimulation of phospholipase D by epidermal growth factor requires protein kinase C activation in Swiss 3T3 cells. The Journal of biological chemistry 270, 3980-3988.

Yoon, M.S., and Chen, J. (2008). PLD regulates myoblast differentiation through the mTOR-IGF2 pathway. Journal of cell science 121, 282-289.

Yuan, T.L., and Cantley, L.C. (2008). PI3K pathway alterations in cancer: variations on a theme. Oncogene 27, 5497-5510.

Zhang, Y., Altshuller, Y.M., Hammond, S.M., Hayes, F., Morris, A.J., and Frohman, M.A. (1999). Loss of receptor regulation by a phospholipase D1 mutant unresponsive to protein kinase C. The EMBO journal 18, 6339-6348.

Zhang, Y., Gao, X., Saucedo, L.J., Ru, B., Edgar, B.A., and Pan, D. (2003). Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. Nature cell biology 5, 578-581.

Zhao, C., Du, G., Skowronek, K., Frohman, M.A., and Bar-Sagi, D. (2007). Phospholipase D2-generated phosphatidic acid couples EGFR stimulation to Ras activation by Sos. Nature cell biology 9, 706-712.

Zheng, Y., Rodrik, V., Toschi, A., Shi, M., Hui, L., Shen, Y., and Foster, D.A. (2006). Phospholipase D couples survival and migration signals in stress response of human cancer cells. The Journal of biological chemistry 281, 15862-15868.

Zipfel, P.A., Brady, D.C., Kashatus, D.F., Ancrile, B.D., Tyler, D.S., and Counter, C.M. (2010). Ral activation promotes melanomagenesis. Oncogene 29, 4859-4864.

Zoncu, R., Efeyan, A., and Sabatini, D.M. (2011). mTOR: from growth signal integration to cancer, diabetes and ageing. Nature reviews Molecular cell biology 12, 21-35.