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The Role of Protein Kinase C in Mammalian Target of Rapamycin Signaling and its Therapeutic Implications in Non-Small Cell Lung Cancer

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Abstract of the Dissertation

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The mammalian target of rapamycin (mTOR) signaling pathway, which integrates extracellular and intracellular signals, plays a key role in cell metabolism, growth and survival; therefore, the proteins in the signaling pathway must be tightly regulated in order to maintain cellular homeostasis. Conversely, dysregulation of key components of mTOR signaling pathway leads to increased cellular growth and proliferation, which is implicated in many human cancers. Protein kinase C (PKC) is a family of serine/ threonine kinases that regulate many cellular processes including proliferation and cell survival. It has been shown to regulate mTOR signaling in some human cancers. However, the mechanism by which PKC regulates mTOR remains unclear. Recently, studies from our lab have shown that sustained activation of PKC induces robust phosphorylation of p70S6K, a well-known substrate of mTORC1, in a delayed pattern, and this

phosphorylation requires classical PKC (cPKC) and phospholipase D (PLD). Importantly, the phosphorylation of p70S6K requires internalization of cPKC and the formation of the cPKC-containing endosomes, a dynamic subset of the slow recycling compartment that depends on PKC and PLD. In addition, we observed that sustained activation of PKC induces mTOR translocation to lysosomes, which appears to require the distinct nPKCs subfamily. Indeed, with PMA treatment, the mTOR- containing lysosomes become very close to the PLD-and PKC-containing endosomes but they remain distinct. These data suggest that sustained activation of PKC activates mTORC1 through a novel and more complicated mechanism. Lastly we found that a subset of non-small cell lung cancer (NSCLC) cell lines which have constitutively active epidermal growth factor receptor (EGFR) tends to have higher protein level of protein kinase c α (PKCα). Interestingly, in these cancer cells, the activation of mTOR is highly dependent on the activity of PKCα and PLD, and these cells utilize this pathway for cell survival. These data have important clinical implication as most NSCLC patients develop secondary mutation in EGFR and resistance to treatment with EGFR inhibitors, and our data provide evidence that targeting PKCα in mutant EGFR-mediated cancers may be used as second line treatment after patients develop the secondary mutation.

Dedication Page

I dedicate my dissertation work to my family and many friends. A special feeling of gratitude to my loving parents whose words of encouragement and push for tenacity ring in my ears. Also to my uncle, thank you for always inspiring me and giving me great suggestion.

I also dedicate this dissertation to my many friends who have supported me throughout the process. I will always appreciate all they have done.

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List of Abbreviations

AMPK	AMP-activated protein kinase
ATII	Angiotensin II
AT1AR	Angiotensin II type 1 receptor
Bis	Bisindolylmalemide I
cPKC	Classical or conventional protein kinase C
DAG	Diacylglycerol
EGFR	Epidermal growth factor receptor
FKBP38	FK506 binding protein 38
GPCR	G-protein coupled receptor
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 1
mtEGFR	mutant EGFR
nPKC	Novel protein kinase C
NSCLC	Non- small cell lung cancer
PA	Phosphatidic acid
PCN	Pericentrion
PKC	Protein kinase C
PI3K	Phosphoinositide 3- kinase
PLC	Phospholipase C
PLD	Phospholipase D
PMA	4β-phorbol-12-myristate-13-acetate
PM	Plasma membrane

P70S6K	p70 Ribosomal S6 kinase
REDD1	Regulated in development and DNA damage responses 1
RTK	Receptor tyrosine kinase
SCLC	Small cells lung cancer
Ser	Serine
Thr	Threonine
TKI	Tyrosine kinase inhibitor
Tyr	Tyrosine
Vacuolar ATPase	V-ATPase
5-HT	5-hydroxytryptamine

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This body of work would not have been done without the support of my families and friends. I would like to take this opportunity to acknowledge and thank these people.

First, and most of all, I would like to thank my parents for their endless love, support and encouragement. They always listen to me and support me without any reservation. When I decided to come to the United States to pursue my Ph.D. degree, they fully respected my decision, even though that they did not want their only kid travel such long distance and to be far away from them. My uncle deserve my wholehearted thank as well. He works in the scientific filed too. When he knew that I want to be a scientist too, he spent a lot time on teaching me how to think like a scientist. He treats me like I am his own daughter and always be there when I need a hand and advice.

I would like to sincerely thank my mentor, Dr. Hannun, for his guidance and support throughout my Ph. D. study, and especially for his confidence in me. Whenever I meet problems in the lab or in my personal life, he encourages and supports me without any hesitation. I would also like to thank Dr. Obeid. I will always remember her smile and encouragement.

To all my friends, thank you for your understanding and encouragement. Your friendship makes my Ph.D. life a wonderful experience. Without your company I could not have such wonderful experience in my Ph.D. study.

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- **Define novel mechanisms of regulation of mammalian target of rapamycin complex 1 (mTORC1) by protein kinase C (PKC) isoenzymes.** The project is designed to understand the mechanisms of activation of mTORC1 by PKC and the emerging PKC/mTOR pathway in oncogenesis and tumor promotion.

- **Determine a novel pathway mediated by PKC and mTOR in mediating survival signaling and oncogenic properties driven by mutant EGFR.** This project is designed to understand the roles of PKC and mTORC1 in the signaling pathway driven by mutant EGFR and to study the mechanistic rationale for novel therapeutic strategies aimed at lung cancers with mutant EGFR.

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Chapter 1

Protein Kinase C: Function, Regulation and its Emerging Roles in Lung Cancers

Abstract

Protein kinase C (PKC) isoforms comprise a family of isozymes that have been implicated in the regulation of a variety of cellular processes, including receptor desensitization and internalization, neurotransmission, cell growth, transcription and immune responses. In normal cells, PKCs play important roles in several signal transduction cascades by relaying information from receptors into a regulated intracellular response. In cancer cells, altered PKC isoforms are observed and often associated with enhanced cancer phenotypes. In this chapter, I summarize the activation and regulation of PKC through receptors and highlight the roles of the PKC isoforms in cancer biology.

1.1. Introduction

The discovery that protein kinase C (PKC) is the target of the tumor-promoting phorbol esters can be tracked back to the early 1980s (1), which catapulted this enzyme to the forefront of research in signal transduction and cancer. In the following years, upon extensive investigation on PKC, the regulation and function of PKC isozymes become clearer. PKC isozymes transduce the multitude of signals from receptors into specific intracellular responses. The increase in concentration of the lipid second messenger, diacylglycerol, and calcium results in the recruitment of most PKC isozymes from the cytosol, where they are maintained in an inactive status, to the plasma membrane, where they become activated (2).

PKC isozymes are divided, based on structures and activation cofactors, into three subgroups: classical, novel and atypical (2,3). With acute stimulation upon agonist binding to G-protein coupled receptors (GPCR) or receptor tyrosine kinase, most PKC isozymes translocate from the cytosol to the plasma membrane (3-5), bringing the isozymes in close proximity to their substrates, like MARCKs (5)and Ezrin(6,7). Recent studies from my lab reported a new mechanism of regulation of PKC involving sustained activation that results in novel translocation of cPKC to a perinuclear compartment with receptors and recycling endosomes(8). The novel mechanism requires activity of cPKC and PLD and also requires endocytosis(9,10). In addition, our data also suggest that sustained activation of cPKC results in activation of mTORC1, which is often dysregulated in pathological conditions, like diabetes and cancers(10). PKC is also involved in the internalization and desensitization of receptors, which plays important roles in the regulation of normal and pathological processes such as cardiovascular hypertrophy (11,12)and the development of cancers(13). In this chapter, I will focus on activation and regulation of PKC via receptors and its role in cancer development.

1.2. Activation and Regulation of PKC through Membrane Receptors

1.2.1. Activation of PKC via G- Protein Coupled Receptor

The largest family of cell surface receptors are the G-protein coupled receptors (GPCRs).

There are hundreds of different GPCR proteins, and they are the largest family of proteins targeted by drug discovery. Angiotensin receptors, one class of G protein-coupled receptor, are important membrane proteins that transduce signal from environment to cells and then induce cellular functions like proliferation, migration and hypertrophy. Angiotensin II type 1 receptor (AT1AR), one kind of angiotensin receptor, is predominantly expressed in cardiovascular cells, which plays key role in the regulation of cardiovascular homeostasis(14). Once activated, the receptor in turn couples to Gq/11 and Gi/o and thus activates phospholipase C and increases the cytosolic Ca2+ concentration, which then activates various intracellular kinases, such as protein kinase C(15). Physiological effects mediated by the AT1AR include vasoconstriction, but irregular Angiotensin II (Ang II) level in blood or abnormal activities of AT1AR may also cause some cardiovascular diseases. Although studies on AT1AR mainly focus on cardiovascular diseases for decade after its cloning, now more and more research implicate that AT1AR is involved in the promotion of tumor-related angiogenesis and growth(16-18).

Upon binding to its ligands, AT1AR stimulates PKC through increasing the concentration of diacylglycerol and calcium. Activated PKC then induced downstream signaling cascades regulating specific cellular responses. It has been shown that in breast epithelial cells activation of AT1AR by Ang II stimulated cell proliferation, which was blocked by cPKC inhibitor, Go6976(15). Our recent study found that sustained activation of cPKC by Ang II resulted in co-sequestration of AT1AR and epidermal growth factor receptor (EGFR) into Rab11 endosomes, which in turn protects EGFR from EGF-induced degradation(19). As AT1AR, PKC and EGFR have been reported to play important roles in cancer biology, these results have implication in AT1AR, PKC and EGFR related mechanisms of oncogenesis and tumor biology. Indeed, studies in cell lines have reported that EGFR escapes from degradative pathway to the recycling compartment, and this contributes to their enhanced malignant phenotype(20).

1.2.2. Activation of PKC via Receptor Tyrosine Kinase

The receptor tyrosine kinase (RTK) family of transmembrane ligand-binding proteins is comprised of 59 members in the human genome. All RTKs have similar structural and functional

characteristics. Most RTKs have an extracellular ligand-binding domain, a transmembrane domain, and an intracellular domain that includes a region with protein-tyrosine kinase activity. Epidermal Growth Factor Receptor (EGFR) is one of the best- studied RTKs. Upon binding with its ligands, activated EGFR undergoes dimerization and autophosphorylation on several tyrosine residues, and then elicits downstream signal transduction cascades leading to cell growth, proliferation and migration. The activation of PKC by EGFR requires the autophosphorylation at Y992 site, which is the binding site for phospholipase C γ, leading to specific cellular responses(21). There are many studies suggesting the important role of PKC in the EGFR signaling pathway especially in cancers. It has been reported that in gliomas, EGFR activates mTORC1 through PKCα, identifying PKCα as key intermediate linking EGFR to mTORC1(22). Our recent results suggest that in non-small cell lung cancer cell lines, constitutively active EGFR (mtEGFR) mutant stimulates mTORC1 via PKCα. Moreover, the cancer cells are highly dependent on the mtEGFR/ PKCα for cell survival under serum starvation.

1.3 PKC-dependent sequestration of molecules to the perinuclear recycling compartment

In addition to the 'classical' signaling pathway for PKC occurs upon activation of either GPCRs or RTK, studies from our lab identified a novel mechanism by which PKC regulates sequestration of membrane proteins and lipids into cPKC-containing endosomes. Upon sustained stimulation of PKC by phorbol esters or serotonin (30- 60 min), PKCα and PKCβII were shown to translocate to a perinuclear region, co-localized with Rab11, a marker of the perinuclear recycling compartment(8,9). Moreover, with sustained activation of PKC, transferin also sequestrated into the same compartment, indicating a broad effect on recycling endosomes(9). Notably, sustained activation of PKC also led to sequestration of several membrane proteins to the cPKC- containing perinuclear endosomes, including caveolin and CD59. In addition, it was also shown that the formation of the cPKC-containing endosomes requires clathrin-dependent endocytosis(9). Interestingly, translocation of cPKC and receptors to the perinuclear endosomes was shown to be dependent on phospholipase D (PLD). Surprisingly, upon stimulation with PMA and serotonin, PLD itself was shown to translocate to the endosomes with cPKC(8,9).

Moreover, our studies showed that sustained activation of cPKC resulted in concentration of several cPKC substrates in the cPKC- containing endosomes, suggesting that the cPKC-containing endosomes might be a potential signaling compartment for cPKC(23). Indeed, we observed that sustained activation of PKC (30- 60 min) led to corresponding robust phosphorylation of p70S6K, a major substrate of mTORC1, which peaked at 30- 60min(23). This time frame matched to the internalization of PKC and the formation of the cPKC-containing recycling endosomes, suggesting an important role the cPKC in the activation of mTORC1 signaling pathway. Importantly, we found that increased PKC activity or expression is important for mTOR activity in cancer cells and the PKC/mTOR pathway is essential to confer oncogenic properties, such as proliferation and migration (10).

1.4. PKC isozymes in Lung Cancer and as Therapeutic Targets for Treatment

In cancer cells, it is well known that PKC isozymes are involved in cell proliferation, survival, invasion, migration, apoptosis and angiogenesis through participating in various cellular signaling pathways, such as Ras/Raf/MEK/ERK or PI3K/Akt/mTOR pathway(22,24-26). In regard to lung cancer, many studies have shown that PKCα plays very important roles in cell proliferation, growth and metastasis. It is reported that PKCα is highly expressed in NSCLC patients with higher level preferentially expressed in adenocarcinoma compared to squamous cell carcinoma(27). In addition, PKCα was shown to interact with discs large homology-1 to promote the migration of NSCLC cells(28). Notably, Chen's lab showed that down-regulation of PKCα by the miR-203 induces apoptosis in A549 cells and targeting PKCα by miR-203 plays significant role in cell proliferation, apoptosis and migration(29).

All these data suggest that PKCα is an important target for treatment of lung cancer, but clinical trials using LY900003, and antisense inhibitor of PKCα in combination with anticancer drugs did not enhance survival in patients with advanced NSCLC(30).

1.5 Conclusion

Though much progress has been made towards understanding the regulation and function of PKC isozymes in lung caner, much remains to be learned about the biology of PKC isozymes and important signaling transduction pathways related with PKC. One of the major challenges is that the regulation and function of PKC is extraordinarily complex and cell-context dependent. In addition, the availability of PKC inhibitors remains small, and most of the inhibitors are not specific. So more studies on biology of PKC and generating more selective inhibitors of PKC needed to be done before targeting PKC isozymes for cancer therapy.

Chapter 2

Sustained Activation of Protein Kinase C Induces Delayed Phosphorylation and Regulates the Fate of Epidermal Growth Factor Receptor

Abstract

It is well established that acute activation of members of the protein kinase C (PKC) family induced by activation of cellular receptors can transduce extracellular stimuli to intracellular signaling. However, the functions of sustained activation of PKC are not well studied. Previous study from our lab has shown that sustained activation of classical PKC isoforms over 15-60 min induced the formation of the pericentrion, a subset of recycling endosomes that are sequestered perinuclearly in a PKC- and phospholipase D (PLD)-dependent manner (9,31). In this study, we investigated the role of this process in the phosphorylation of EGFR on threonine 654 (Thr-654) and in the regulation of intracellular trafficking and fate of epidermal growth factor receptor (EGFR). Sustained stimulation of the angiotensin II receptor induced translocation of the EGFR to the pericentrion, which in turn prevents full access of EGF to the EGFR. These effects required PKC and PLD activities, and direct stimulation of PKC with phorbol esters was sufficient to reproduce these effects. Furthermore, activation of PKC induced delayed phosphorylation of EGFR on Thr-654 that coincided with the formation of the pericentrion and which was dependent on PLD and endocytosis of EGFR. Thus, Thr-654 phosphorylation required the formation of the pericentrion. On the other hand, using a T654A mutant of EGFR, we find that the phosphorylation on Thr-654 was not required for translocation of EGFR to the pericentrion but was required for protection of EGFR from degradation in response to EGF. Taken together, these results demonstrate a novel role for the pericentrion in the regulation of EGFR phosphorylation, which in turn is important for the fates of EGFR. (This study has already published in Plos One)

2.1. Introduction

Protein kinase C (PKC) is a family of enzymes implicated in numerous cellular processes including proliferation, migration and cell survival. Currently, there are eleven known PKC isoforms grouped into 3 subfamilies according to their domain structure and activation. Classical PKCs (cPKCs; α, βI, βII and γ) have functional C1 and C2 domains, and are activated by phosphatidylserine (PS), diacylglycerol (DAG) and calcium(32). The novel PKCs (nPKCs;δ, ε, η and θ) are PS- and DAG-dependent but are calcium-independent as they have truncated C2 domains(32). Differing from both cPKCs and nPKCs, the atypical PKCs (ι, λ, ζ) have truncated C1 domains and no C2 domain (33) and are also independent of DAG activation. Notably, tumor promoting phorbol esters, such as 4β-phorbol-12-myristate-13-acetate (PMA), were found to directly activate both cPKC and nPKC isoforms by mimicking DAG (34). In the classical paradigm, cPKCs are acutely activated upon agonist binding to tyrosine kinase or G-protein coupled receptors (GPCR) such as the angiotensin II type 1A receptor, which results in stimulation of phospholipase C (PLC) isoforms. PLC subsequently hydrolyzes phosphatidylinositol 4,5 bisphosphate to produce inositol 1,4,5- triphosphate (IP3), which releases calcium from intracellular stores, and DAG (35). This results in translocation of cPKCs from the cytosol to the plasma membrane within 60 seconds (36), bringing it in close proximity to its substrates.

In addition to the above well-established paradigm of acute activation and translocation of cPKCs, it was previously reported by our group that sustained activation of cPKCs, both by PMA or by activation of GPCRs such as the serotonin receptor, resulted in internalization of cPKCs from the plasma membrane and their translocation to a perinuclear compartment involving recycling endosomes that become sequestered around pre-existing Rab11 endosomes (8,31). Additional results revealed that this is a dynamic compartment, requiring both PKC and PLD activities and dependent on clathrin-mediated endocytosis (37). We termed these sequestered PKC- and PLD-dependent endosomes the pericentrion. Furthermore, formation of the pericentrion did not occur at temperatures below 32 °C, which distinguished the process from general endocytosis (9). Functionally, formation of the pericentrion also induced sequestration of recycling molecules such as transferrin, some membrane receptors (e.g. 5-HT receptor) and

membrane lipids (ganglioside GM_1) (8). Besides sequestration of different molecules, additional studies have shown that formation of the pericentrion also coincided with the phosphorylation of a subgroup of PKC substrates including Rab11, S6 kinase and transferrin receptor. (23).

The epidermal growth factor receptor (EGFR) is one of the best-studied tyrosine kinase receptors. EGFR can be activated by numerous ligands including neuregulins (38) but, to date, transforming growth factor-α and epidermal growth factor (EGF) are the best studied. The binding of ligands to the extracellular domain of EGFR induces autophosphorylation on several tyrosine residues of the receptor with each site mediating specific functions of EGFR regulation (39-43). For example, phosphorylation at tyrosine 1045 (Tyr-1045) is reported to play a key role in endocytosis and translocation of the receptor to the lysosome for degradation (44-46) and autophosphorylation at tyrosine 1068 (Tyr-1068) is a major event for EGFR activation (47). In addition to autophosphorylation, it is also known that PKCs can phosphorylate EGFR on Thr-654, and this may protect the EGFR from degradation in the lysosome (45). In a recent study, we showed that sustained treatment with serotonin (5-HT) led to sequestration of EGFR in the perinuclear region, and this process was dependent on cPKC and PLD activities, indicative of its localization in the pericentrion (8). Consequently, it became of great interest to determine the mechanistic relationship between translocation to the pericentrion, phosphorylation of EGFR on Thr-654, and diversion of the EGFR into the recycling pathway.

Here, using HEK293 cells as a model system, we provide evidence that the phosphorylation of EGFR on Thr-654 is a delayed process that requires formation of the pericentrion. Furthermore, we show that PMA induces sequestration of EGFR from the plasma membrane to the perinuclear recycling endosome in a cPKC- and PLD-dependent manner and that this sequesters EGFR from EGF binding. Notably, the protection of EGFR from EGF binding required the phosphorylation of EGFR on Thr-654. Collectively, these results identify a novel role for the pericentrion in the regulation of EGFR phosphorylation and intracellular trafficking.

2.2 Materials and Methods

2.2.1. Materials

Minimal essential medium (MEM) was from Invitrogen. The HEK293 cell line was purchased from American Type Culture Collection. HEK293 cells with stable expression of $AT_{1A}R$-GFP （angiotensin II type 1A receptor) that were previously characterized (48) were gifts from Dr. Thomas A. Morinelli (Medical University of South Carolina, Charleston, SC). 4-Phorbol 12-myristate-13-acetate (PMA), GÖ6976 and Bisindolylmaleimide I (Bis) were purchased from Calbiochem. Anti EGFR, and Phospho-Thr-654 EGFR antibodies were from Upstate Biotechnology (Lake Placid, NY). Phospho-Tyr-1045 EGFR and phospho-Tyr-1068 EGFR antibodies were from Cell Signaling. Na^+K^+ATPase antibody was from Abcam. β-Actin antibody was from Sigma. Other antibodies were from Santa Cruz. Alexa Fluor 555 secondary antibody was from Invitrogen. DRAQ5 was from Biostatus Limited. FIPI was a gift from Dr. Michael Frohman (Stony Brook University, School of Medicine). Serotonin, epidermal growth factor, angiotensin, and all other chemicals were from Sigma.

2.2.2. Cell Culture

HEK293 cells were grown in MEM supplemented with 10% (v/v) fetal bovine serum. The HEK293 cells with stable expression of $AT_{1A}R$-GFP were grown in MEM containing 10% (v/v) fetal bovine serum (FBS) and 700 μg/ml Geneticin. All cells were grown in a 5% CO_2 incubator at 37°C.

2.2.3. Plasmids

All plasmids were made by standard protocols. HA-tagged mutants of PLD1 (K898R) and PLD2 (K758R) were gifts from Guangwei Du (Stony Brook University, New York, NY). Human EGFR and the mutant T654A EGFR were subcloned into pEGFP-N1 vector by polymerase chain reaction (PCR). These constructs were then used to perform cell studies. The EGFR PCR products were generated with a 5’AAAAAAAACCCAAGCTTGCGATGCGACCCTCCGGGACGGCCG GG primer

containing a HindIII site and a 3'TCGGGGTACCTTTGCTCCAATAAATTCACTGC TTTG primer that was minus the stop codon sequence and containing a KpnI site. The amplified EGFR products were then subcloned into the pEGFP-N1 vector and maxi-prepped. The vectors were then sequenced for confirmation.

2.2.4. Transient Transfection, Indirect Immunofluorescence, and Confocal Microscopy

Cells were plated on 35-mm confocal dishes (MatTek) at a density 3 to 5 $\times 10^5$ cells/dish. After 24 hours, Lipofectamine 2000 (Invitrogen) was used for transient transfection following the manufacturer's recommendation. Transfected cells were grown in normal medium with 10% FBS for 24 hours and then starved with medium with 0.1% bovine serum albumin (BSA) for 5 hours, followed by treatments. Cells expressing green fluorescent protein (GFP) were fixed with 3.7% formaldehyde for 10 minutes and then analyzed by confocal microscopy. For indirect immunofluorescence, the procedure was the same as described before (8). All images were taken by LSM 510 Meta from Zeiss (ZESS 510) and pictures are representative of three fields examined from three independent experiments.

2.2.5. Immunoblotting

Protein samples were boiled for 15 min in LDS sample buffer (Invitrogen, NuPAGE®) and separated by 4-20% polyacrylamide gel (BioRad, Criterion Tris-HCl Gel). Proteins were transferred to Nitrocellulose membranes (BioRad) and the membranes were blocked in PBST with 5% nonfat dried milk for 1 hour, followed by washing with PBST and then further incubation with primary antibody overnight at 4 °C. All primary antibodies were diluted to 1: 1000 in PBST with 2% BSA. On the following day, the blots were washed with PBST and incubated with secondary antibody in PBST with 5% nonfat dried milk for 1 hour. After washing, proteins were detected by using enhanced chemiluminescence reagent (Pierce).

2.2.6. Statistics

Statistical significance was calculated with student's t-test or by two-way ANOVA with Bonferroni Post test where appropriate. A p-level of below 0.05 was considered to be statistically significant.

2.3. Results

2.3.1 Sustained AT-II treatment induces co-sequestration of $AT_{1A}R$ and EGFR in the pericentrion and protects EGFR from EGF-induced degradation.

In previous study from our lab, our lab reported that sustained stimulation with serotonin (5-HT) led to co-sequestration of the 5-HT receptor and EGFR to the pericentrion (8). To determine if this effect extends to other GPCRs, the effects of AT-II on $AT_{1A}R$ and EGFR were examined. Utilizing HEK cells stably expressing $AT_{1A}R$ as a model system, the effects of AT-II on receptor localization were initially determined. As can be seen, $AT_{1A}R$ predominantly localized on the plasma membrane in control cells, but following prolonged AT-II treatment (60 min), the majority of $AT_{1A}R$ had translocated to a perinuclear region where it partly co-localized with Rab11, a marker of the perinuclear recycling compartment (**Fig.1A**). Next, the effects of AT-II on localization of both AT-II and EGFR were assessed. Again, most $AT_{1A}R$ and EGFR localized on the plasma membrane in control cells that were serum starved. As seen above, AT-II treatment induced translocation of the $AT_{1A}R$ to the pericentrion. Importantly, AT-II induced translocation of both receptors showing significant co-localization in the perinuclear compartment (**Fig.1B**).

To determine if this sequestration in the pericentrion had effects on the fate of EGFR, the effects of EGF on EGFR with pretreatment with AT-II were determined. As can be seen, EGF treatment induced a significant loss of the EGFR protein; however, with the pretreatment of AT-II, the EGF didn't induce the loss of EGFR protein (**Fig.1C**). Collectively, these results show that AT-II induces translocation of both the $AT_{1A}R$ and the heterologous receptor, EGFR to the pericentrion with the functional consequence of inhibiting the loss of EGFR induced by EGF.

2.3.2. AT-II-induced sequestration of the EGFR and inhibition of EGF-induced loss require the formation of the pericentrion.

The above results show that AT-II induces sequestration of EGFR in the pericentrion and inhibits it from the loss induced by EGF. Therefore, it became important to determine if the pericentrion is necessary for the inhibition. To explore this, PMA was used as a primary inducer of the pericentrion, as reported both by our laboratory and that of Exton. For this, two different time points of PMA stimulation were used – 5 min, associated with PKC localization to the PM and 60 min, associated with formation of the pericentrion. As before, the majority of EGFR basally localized at the plasma membrane. However, after 5 min of PMA treatment, some EGFR had begun to internalize and partially co-localized with early endosome marker EEA1. In contrast, by 60 min of PMA treatment, the majority of EGFR had translocated to the perinuclear region and did not significantly co-localize with EEA1, suggesting that the internalized EGFR has left the early endosomes (**Fig 2A**).

Our lab has previously characterized the pericentrion as a dynamic compartment requiring PKC and PLD activity for its formation and therefore, the roles of PKC and PLD in regulating the cellular fate of EGFR were examined. As can be seen, pre-treatment with the cPKC inhibitor Gö 6976 (1h) prevented PMA-induced translocation of EGFR to the pericentrion (**Fig. 2B**). Likewise, following inhibition of PLD by pretreatment with 0.4% 1-butanol, EGFR was not sequestered in the perinuclear compartment, but was present in large vesicles dispersed throughout the cell (**Fig 2B**). Thus, these results suggest that both cPKC and PLD activities may be required for EGFR sequestration induced by PMA. To further confirm that EGFR translocates to the pericentrion with sustained activation of PKC, we performed co-localization studies of EGFR with Rab11, previously identified as a marker of the pericentrion (**Fig. 2C**). As can be seen, sustained PMA stimulation induced a strong co-localization of EGFR with Rab11 but, importantly, this was completely distinct from the lysosomes (**Fig. 2D**). Indeed, although PMA induced clustering of EGFR and LAMP1 within the same area, there was no obvious overlap in signal. Taken together, this suggests that PMA induces trafficking of the EGFR to the pericentrion (the PKC-containing subset of Rab11 positive recycling endosomes) but does not target EGFR to the lysosomes.

As PMA induced EGFR localization to the pericentrion, this suggested that it might also protect EGFR from degradation induced by EGF. To assess this, cells were pre-treated with PMA prior to stimulation with EGF. As can be seen, PMA treatment did indeed lead to inhibition of the loss of EGFR induced by EGF (**Fig 2E**). Importantly, both the Gö 6976 (cPKC inhibitor) and bisindolylalemide (cPKC and nPKC inhibitor) prevented the ability of PMA to induce the inhibition (**Fig 2E**). Similarly, inhibition of PLD with 1-butanol, which has been shown to disrupt the pericentrion (49), also prevented PMA-induced protection of the EGFR (**Fig 2F**). To confirm that PMA and AT-II were regulating EGFR at the protein level, and not through transcriptional mechanisms, the effects of PMA and AT-II on EGFR mRNA levels was assessed by qRT-PCR. As shown in Figure 2G, PMA had a small but statistically insignificant decrease in EGFR mRNA whereas AT-II treatment had no major effect on EGFR mRNA. This confirms that sustained PKC activation regulates EGFR at the protein level rather than through transcriptional regulation.

2.3.3. AT-II induces alterations in EGFR phosphorylation

Previous research has reported that phosphorylation of EGFR at various residues is important for regulating its trafficking. Indeed, PMA-induced phosphorylation of EGFR on Thr-654 was reported to change its fate from the lysosomes to the recycling endosomes (45). In contrast, phosphorylation of EGFR on tyrosine 1045 (Tyr-1045) was found to be necessary for binding to c-Cbl, receptor ubiquitination, and degradation (50). As the pericentrion is a subset of recycling endosomes and is required for the AT-II-induced inhibition, it became important to determine the role of these phosphorylation sites in this process. Initially, the effects of AT-II on phosphorylation of EGFR at Thr-654 and Tyr-1045 were determined. As can be seen (**Fig. 3A**), there was a basal phosphorylation of Thr-654 in unstimulated cells that was strongly increased by AT-II treatment. In contrast, basal phosphorylation of EGFR at Tyr-1045 was minimal and was sharply increased by EGF treatment, consistent with induction of EGFR degradation. However, while AT-II had no effect on basal Tyr-1045 phosphorylation, pretreatment of cells with AT-II partially inhibited the phosphorylation of Tyr-1045 induced by EGF (**Fig 3B**). As Tyr-1045 phosphorylation is important for EGFR degradation, this could account for the

observed effect of AT-II on the inhibition of the loss of EGFR. If this were the case and, given results suggesting that EGFR sequestration in the pericentrion is required to prevent EGFR degradation (**Fig. 2E**), we reasoned that the effect of AT-II on Tyr-1045 would be sensitive to inhibitors of pericentrion formation; consequently, cells were treated with inhibitors of cPKC (Gö6976) and PLD (FIPI) (**Fig. 3C**). The results showed that inhibition of either PKC or PLD (51,52) did not prevent the effects of AT-II on Tyr-1045 phosphorylation, thereby suggesting that Tyr-1045 phosphorylation occurs independently of the pericentrion.

Finally, it has also been shown that GPCR ligands can stimulate the cleavage of pro-forms of high efficacy EGFR ligands that activate the receptor but induce lower levels of EGFR degradation (53). To assess this possibility, the effect of AT-II on phosphorylation of the EGFR residue Tyr-1068, a major autophosphorylation site on EGFR that functions as a binding site for the Grb2 adaptor protein and a marker of receptor activation were assessed. Moreover, to determine if the pericentrion is involved in any effects, the dependency of this process on PKC and PLD activities was determined (**Fig. 3D**). The results showed that, as with Thr-654, AT-II increased the Tyr-1068 phosphorylation consistent with EGFR transactivation; however, while inhibition of cPKCs with Gö6976 inhibited this phosphorylation, PLD inhibition with 1-Butanol had no effect (**Fig 3D**). This indicates that transactivation of EGFR by AT-II is PKC- but not PLD- dependent. Collectively, these results suggest that, unlike AT-II-induced protection, phosphorylation of the EGFR at both Tyr-1045 and Tyr-1068 are independent of PLD and, by extension, the pericentrion.

2.3.4. The pericentrion is required for Thr-654 phosphorylation on the EGFR.

Thus far, the results demonstrated a role for the pericentrion in the AT-II induced protection of EGFR, and suggested that this is not through effects on Tyr-1045 phosphorylation. As previous results have reported that phosphorylation of EGFR at Thr-654 is PKC-mediated and is important for regulating EGFR fate (45), it became important to determine the role of the pericentrion in this process. Initially, the effects of PMA on Thr-654 phosphorylation were determined. As can be seen (**Fig. 4A**), PMA initiated phosphorylation of Thr-654 at 10 min with

levels being sustained for the course of the experiment (Fig. 4A). Importantly, this time course of phosphorylation is significantly later than initial events mediated by PKC at the plasma membrane (which occurs within 30-60 seconds) and is more coincident with formation of the pericentrion.

To further implicate the pericentrion in regulating Thr-654 phosphorylation, we reasoned that disrupting pericentrion formation would prevent the effects of PMA on Thr-654. As noted above, we have previously established the pericentrion as cPKC and PLD-dependent. (9,31,37). Consistent with this, inhibition of cPKC (Gö6976) and PLD (1-Butanol) prevented PMA phosphorylation of Thr-654 (Fig 4B). We have also found that clathrin-dependent endocytosis is required for PMA induction of the pericentrion. Accordingly, to inhibit clathrin-dependent endocytosis, cells were either depleted of potassium or preincubated in the presence of high concentration of sucrose (54). As with cPKC and PLD inhibitors, both these treatments inhibited PMA-induced Thr-654 phosphorylation (**Fig 4B**). This demonstrates that internalization of EGFR and PKC is required for Thr-654 phosphorylation in response to PMA. In order to corroborate the effects of PLD inhibition and to define the role of individual PLD isoforms in this phosphorylation, HEK293 cells were transfected with dominant negative constructs of PLD1 and PLD2 – both of which have been shown to inhibit pericentrion formation (37). The results show that, as with the pericentrion, both isoforms are required for Thr-654 phosphorylation induced by PMA (**Fig 4C**).

Data above with AT-II suggest that, unlike Thr-654, regulation of EGFR phosphorylation on Tyr-1045 is independent of the pericentrion. To further confirm this, the effects of PMA on Tyr-1045 phosphorylation were determined. Strikingly, effects observed were very rapid with PMA inhibiting EGF-induced Tyr-1045 phosphorylation with as early as 2 min of pretreatment (**Fig 4D**). This is in sharp contrast to the delayed effect on Thr-654, and suggests that this inhibitory effect of PMA precedes formation of the pericentrion. To confirm this, the involvement of cPKCs and PLD in the effects of PMA on Tyr-1045 was studied. As with AT-II, there was some recovery, albeit modest, of Tyr-1045 phosphorylation following inhibition of cPKC; importantly, no effect of the PLD inhibitors 1-But or FIPI on Tyr-1045 phosphorylation levels was observed (**Fig 4E**), confirming that PMA effects on Tyr-1045 do not require PLD or

the pericentrion, and suggest that perhaps nPKCs and not cPKCs mediate this effect of PMA on Tyr-1045 phosphorylation. Collectively, these data show a requirement for PLD in phosphorylation of a cPKC substrate and demonstrate that the pericentrion is essential for PKC-mediated phosphorylation of Thr-654 but not Tyr-1045, and define compartment-specific phosphorylation events for EGFR.

Finally, the data above suggest that AT-II is transactivating the EGFR, as evidenced by increase Tyr-1068 phosphorylation. However, it is unclear if transactivation of the receptor is necessary for sequestration in the pericentrion. To analyze this, the effects of PMA stimulation on Tyr-1068 phosphorylation were examined. As shown in Figure 4F, acute EGF induced a strong increase in Tyr-1068 phosphorylation consistent with EGFR activation. However, PMA had no significant effect suggesting that PMA does not induce transactivation. Furthermore, pretreatment with PMA completely blunted EGF-induced phosphorylation of Tyr-1068, indicating that sequestration of the EGFR in the pericentrion renders the receptor inaccessible to EGF.

2.3.5. Phosphorylation of Thr-654 is required for PMA-induced protection of EGFR away from EGF but not sequestration of the EGFR.

The above results suggest that sequestration, Thr-654 phosphorylation, and protection of EGFR induced by AT-II are all pericentrion-dependent. Therefore, it became important to determine the mechanistic interactions of these effects and, more specifically, if phosphorylation of Thr-654 is essential for either protection or sequestration, and if so, then what is the mechanistic order of these events. To investigate this, a non-phosphorylatable EGFR-T654A mutant (TA-EGFR-GFP) was generated, and the effects of PMA on trafficking of this mutant were compared to wild-type EGFR (WT-EGFR-GP). As seen above, PMA induced translocation of WT-EGFR-GFP to the perinuclear region; strikingly, this was also observed for the mutant TA-EGFR-GFP to the perinuclear region (**Fig 5A**), demonstrating that Thr-654 phosphorylation is not required for sequestration, and also consistent with the results suggesting that sequestration of EGFR to the pericentrion is necessary for phosphorylation. Importantly, EGF was able to

induce loss of TA-EGFR as with WT-EGFR, and with pretreatment of PMA EGF was able to induce the loss of TA-EGFR mutant (**Fig 5B).** This confirms that Thr-654 phosphorylation is not necessary for its translocation to the pericentrion but is essential for the protection. Thus, the formation of the pericentrion is required for phosphorylation on T654, which in turn is required for the protection of EGFR from accessed by EGF (**Scheme 1**).

2.4. DISCUSSION

The pericentrion is a dynamic subset of recycling endosomes formed upon sustained stimulation with PMA or GPCR ligands, and requiring both PKC and PLD activities. However, to date, the function of the pericentrion in GPCR signaling has remained unclear. Here, we have established a novel role for the pericentrion in regulating EGFR phosphorylation, its intracellular trafficking and cellular fate. This could be of particular relevance in pathologies where cPKC and/or PLD activity are increased.

To date, a number of studies have implicated PKC in the regulation of EGFR including its transactivation (55,56) and degradation (57-59). Despite this, the mechanisms underlying these processes have remained unclear. The results presented herein now establish a crucial role for the pericentrion in sequestration of the EGFR from access by EGF. These conclusions come from several lines of evidence. Firstly, sustained activation of PKC by AT-II inhibited the loss of EGFR induced by EGF treatment coincident with co-sequestration of EGFR and the $AT_{1A}R$ in the pericentrion. Importantly, both cPKC and PLD activities were required for both EGFR protection and sequestration, consistent with our previous studies characterizing the pericentrion as cPKC- and PLD-dependent (31,37) Moreover, disruption of clathrin-mediated endocytosis, previously shown to disrupt the pericentrion, also abrogated the protective effects of AT-II and PMA on EGFR. Finally, sustained PKC activation with PMA was sufficient to induce EGFR sequestration to the pericentrion and reduced EGF access, also in a cPKC and PLD-dependent manner. These results are also highly consistent with and build upon our previous study demonstrating co-sequestration of both the 5-HT receptor and the EGFR in the pericentrion following 5-HT stimulation (8). By extending these previous findings, it is evident that both

translocation of GPCRs to the pericentrion and the heterologous sequestration of other receptors are emerging as more generalized roles for sustained activation of cPKCs.

Previous research has reported that phosphorylation of EGFR at various residues is important for regulating its trafficking. Indeed, PMA-induced phosphorylation of EGFR on Thr-654 was reported to change its fate from the degradative pathway to the recycling endosome (45). An important conclusion from the current study emanates from the observation that phosphorylation of EGFR on Thr-654 shows delayed kinetics, and the results implicate the formation of the pericentrion in regulating EGFR phosphorylation by PKC. Thus, EGFR phosphorylation on Thr-654 was prevented by inhibiton of cPKC, PLD and endocytosis. These results place EGFR in a newly appreciated subset of PKC substrates that are phosphorylated with delayed kinetics (23). However, this is clearly residue specific as the pericentrion was not required for effects of AT-II or PMA on Tyr-1045 or Tyr-1068. Indeed, results here place phosphorylation of both these residues as upstream of the pericentrion suggesting they likely are not important for the observed protective effect. In agreement with this, our results demonstrate that phosphorylation of Thr-654 is necessary for the EGFR protection – as evidenced by the loss of protection of the TA-EGFR mutant compared to WT-EGFR. However, in contrast to this, the TA-EGFR mutant was able to translocate to the pericentrion equally as well at WT-EGFR. This suggests that phosphorylation of Thr-654 is not a prerequisite for entry into the recycling endosomes but may be crucial for sequestering EGFR in the slow recycling pathway. These data also disclose a sequence of events whereby the EGFR is first translocated to the pericentrion, is phosphorylated on Thr-654, and is then protected from degradation by reducing the access of EGF to the EGFR (**Scheme 1**)

There are several implications from these results, specifically when considering EGFR related mechanisms of oncogenesis and tumor biology, or pathologies wherein PKC and PLD activities are increased. For example, studies in breast cancer cell lines have reported that EGFR escapes from the degradative pathway to the recycling compartment, and that this contributes to their enhanced malignant phenotype (20,60). Moreover, separate studies of breast cancer cell lines have found overexpression of cPKCs and implicated them in cell growth and proliferation (61,62). Additionally, enhanced PLD activity in breast cancer is reported to correlate with

increased invasion, migration and proliferation (63-66). These studies suggest the intriguing possibility that, in these breast cancers, highly activated cPKCs induce the formation of the pericentrion, which facilitates the proliferation and migration of the cancer cells by sequestering and protecting EGFR from accessed by EGF. This could also be true for some non-small cell lung cancers in which cPKCs are highly expressed and the downregulation of EGFR is impaired (27,67,68). These possibilities are currently undergoing further study in our laboratory.

In conclusion, the results reveal a novel role for the pericentrion in regulating EGFR phosphorylation, intracellular trafficking, and fate by protecting EGFR from accessed by EGF. Phosphorylation of EGFR on Thr-654 was pericentrion- dependent and was required for the protection of EGFR. In many cancers, EGFR evades degradation by entering the recycling pathway, and this invites a role for PKC and the pericentrion in these EGF-induced oncogenic properties. (This work has been already published)

Chapter 3

Sustained PKCβII Activity Confers Oncogenic Properties in A Phospholipase D and mTOR-dependent Manner

Abstract

Protein kinase C (PKC) is a family of serine/threonine kinases implicated in a variety of physiological processes. We have previously shown that sustained activation of the classical PKCα and -βII induces their phospholipase D (PLD)-dependent internalization and translocation to a subset of the recycling endosomes defined by the presence of PKC and PLD (the pericentrion) (69), resulting in significant differences in phosphorylation of PKC substrates(23). Here, we have investigated the biological consequences of sustained PKC activity and the involvement of PLD in this process. We find that sustained activation of PKC results in activation of the mammalian target of rapamycin (mTOR)/S6 kinase pathway in a PLD- and endocytosis-dependent manner with both pharmacologic inhibitors and siRNA implicating the PLD2 isoform. Importantly, dysregulated overexpression of PKC-ßII in A549 lung cancer cells was necessary for the enhanced proliferation and migration of these cancer cells. Inhibition of PKC-ßII with enzastaurin reduced A549 cell proliferation by >60% (48h) and migration by >50%. These biologic effects also required both PLD activity and mTOR function with both the PLD inhibitor FIPI and rapamycin reducing cell growth by >50%. Reciprocally, forced overexpression of wild-type PKC-ßII, but not an F666D mutant that cannot interact with PLD, was sufficient to enhance cell growth and increase migration of non-cancerous HEK cells; indeed, both properties were almost doubled when compared to vector control and PKC-F666D-overexpressing cells. Importantly, this was also dependent on both PLD and mTOR activity. In conclusion, these data define a PKC-driven oncogenic signaling pathway that requires both PLD and mTOR, and suggest that inhibitors of PLD or mTOR would be beneficial in cancers where PKC overexpression is a contributing or driving factor.

3.1. Introduction

In the current paradigm, ligands of G protein-coupled (GPCR) or tyrosine kinase receptors activate phospholipase C and the hydrolysis of phosphatidylinositol-4,5-bisphosphate to produce DAG and inositol-1,4,5-trisphosphate, which mediates intracellular calcium release. This coordinated action leads to translocation of PKC to the plasma membrane (PM) within seconds of receptor activation (69,70) allowing phosphorylation of local PKC substrates and activation of downstream signaling. However, this process is short-lived as PKC returns to the cytosol within 2-5 min, correlating with metabolism of DAG (71). In contrast, sustained activation of the cPKCs α and ßII by PMA (31) or GPCRs (8) induces their translocation through the endosomal system to a juxtanuclear region termed the pericentrion (PCN), composed of a subset of recycling endosomes that forms in a PKC dependent manner and that contains both cPKCs and PLD. Notably, this is accompanied by co-internalization and sequestration of several recycling membrane proteins and lipids (8). Mechanistically, unlike acute translocation to the PM, formation of the PCN requires PKC activity. Moreover, inhibition of phospholipase D (PLD) and both clathrin- and caveolae-dependent endocytosis will also prevent the formation of PCN (8,9,31,72).

Given these emerging distinctions between acute activation of PKC and effects of sustained activation on internalization and endocytosis, a major focus of our studies has been to understand the functional consequences of sustained activation of PKC. To this end, we have implicated this process in redirecting the fate of some receptors, such as the thrombin receptor, from the degradative endolysosomal pathway to the recycling pathway. More recently, we demonstrated that phosphorylation of PKC substrates was temporally concurrent with perinuclear localization of PKC and was prevented by both PKC and PLD inhibitors (23). One such candidate substrate with delayed phosphorylation is the p70-S6 kinase (S6K), the best characterized substrate of the mammalian target of rapamycin complex 1 (mTORC1). Biologically, the mTORC1/S6K axis modulates cell proliferation through regulating protein synthesis and the cell cycle(23,73-75) and can regulate cell migration through effects on the cytoskeleton(76). Notably, these processes can also be regulated by a variety of other signaling proteins and second messengers including PKC (77).

In cancer, the function of PKC is very complex, mostly due to its activation by many different signals and its function in many pathways involved in cell transformation. Indeed, increased PKC levels have been associated with malignant transformation in many different cell lines (78). Additionally, PKC regulates at least one step in metastasis by increasing tumor attachment to lung tissues (79). At the molecular level, changes in subcellular PKC localization lead to a reduction in cell adhesion and promoting the transition from the normal to malignant cell phenotype (80). Finally, several studies have reported PKC as an upstream activator of p70-S6K (81,82). However, the functional consequences of PKC activation of S6K or the mechanisms involved have remained poorly defined.

In this study, we have investigated the role of sustained PKC activation and internalization in regulating the mTORC1/S6K pathway and if this is relevant for imparting oncogenic properties. We find that increased PKC expression is both necessary and sufficient to enhance oncogenic properties of cells. Importantly, this occurs in a manner dependent on PLD activity and mTOR, thus defining a novel pathway for cancer cell regulation. .

3.2 Materials and Methods

3.2.1 Materials

All cell lines were from the American Tissue Culture Collection (Manassas, VA). Culture media (MEM, DMEM, RPMI), Lipofectamine 2000, Lipofectamine LTX, Lipofectamine RNAiMAX, and geneticin solution (G418) were from Life Technologies (Grand Island, NY). PMA, Gö6976, enzastaurin were from EMD Millipore (Billerica, MA) and rapamycin from Cayman Chemical (Ann Arbor, MI). Anti-phospho-p70S6K (Thr389), phospho-4EBP1 (Thr37/46), S6K and 4E-BP1, TSC2, mTOR, Akt and PLD1, HA antibodies were from Cell Signaling Technology (Danvers, MA). Anti- PLD2 and actin antibodies were purchased from Sigma Aldrich (St. Louis, MO). Anti-PKC β antibody was obtained from BD Transduction (San Jose, CA). Anti- PKCβII antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). siRNA for PLD1 (Cat# L-009413-00) and PLD2 (Cat# L-005064-00) were purchased from Thermo Scientific (Pittsburgh, PA). PKCβ siRNA was from Santa Cruz Biotechnology (Santa Cruz, CA). The inhibitors specific for PLD1 (VU0359595 (EVJ)) and PLD2 (VU0364739

(JWJ)) were a kind gift from Dr. Alex Brown (Vanderbildt University, TN). FIPI and all other chemicals were from Sigma Aldrich (St. Louis, MO).

3.2.2. Cell Culture and Counting

Generation of HEK cells stably expressing PKCβII-WT was described previously (12). Following selection, cells were sorted by flow cytometry to collect the upper 20% bright GFP population. Stable cell lines were maintained in standard growth media (10% FBS MEM) supplemented with 0.4mg/ml G418. A549 and HeLa cells were cultured in 10% FBS DMEM. For cell counting, cells were trypsinized from culture dishes and 10µl of cell suspension was mixed with Trypan blue in a 1:1 ratio (v:v) for 2 minutes. Cell number was determined using the Biorad TC10 automated cell counter.

3.2.3. Inhibitor and Agonist Treatment

When required, cells were starved in media containing 0.1% BSA overnight. Stock solutions of PMA and inhibitors were prepared in DMSO. PMA was used at 100nM, Gö6976, FIPI, enzastaurin were used at 3µM for 1h prior to treatment and rapamycin was used at 100nM. For long-term culture experiments, inhibitors were added immediately after media at the start of the experiment. When media was changed (every 48h), fresh inhibitors were added.

3.2.4. siRNA Transfection

siRNAs were transfected into HEK cells with Lipofectamine RNAiMAX following the manufacturer's recommendation. Transfected cells were grown in medium with 10% FBS for 48 hours then followed with treatment.

3.2.5. Immunofluorescence

Cells were plated in 35-mm glass-bottomed culture dishes (MatTek) at a density of 5 x 105 cells/dish and incubated overnight. Following treatment, cells were washed once with PBS and fixed with fresh 4% paraformaldehyde for 10 min. After 3 x PBS washes, cells were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes, washed with PBS and blocked with 2% human serum for 1h at room temperature. After incubation with primary antibodies (in 2% serum, overnight at 4°C), cells were incubated with fluorescent dye conjugated secondary antibody and visualized using a laser-scanning confocal microscope (TCS SP8, Leica).

3.2.6. Proximity Ligation Assay

HeLa cells (100K) were plated on 35-mm confocal dishes (MatTek). After 24h, cells were transiently transfected as shown using Lipofectamine LTX (Invitrogen) according to manufacturer's instruction. After 24h growth in full medium, cells were serum starved (DMEM + 0.1% BSA) for 5h followed by treatment with vehicle or PMA as shown. Cells were washed once with PBS and fixed with fresh 4% paraformaldehyde for 10 min. After 3 x PBS washes, cells were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes, washed with PBS and blocked with 2% human serum for 1h at room temperature. After incubation with primary antibodies (in 2% serum, overnight at 4°C), cells were incubated with oligonucleotide labeled secondary antibodies. The PLA assay was performed according to manufacturer's protocol (Olink Bioscience, Uppsala, Sweden) The signal from each detected pair of PLA probes, visualized as an individual fluorescent spot, was acquired with a Leica TCS SP8 microscope. PLA signals were counted and quantified as shown.

3.2.7. Preparation of protein lysate and immunoblotting

Following treatment, cells were washed twice with cold PBS and directly lysed in Laemelli sample buffer. Lysates were sonicated for 10s and centrifuged at 12,000 rpm for 10 min. Samples were boiled for 5-10 min, and proteins were separated via SDS-PAGE (4-15%, Tris-HCl) using the Bio-Rad Criterion system. Proteins were transferred to nitrocellulose

membranes and blocked for at least 1 h with 5% nonfat milk in PBS/0.1% Tween 20 (PBS-T). Membranes were incubated with primary antibodies diluted 1:1000 or 1:3000 β-actin at 4°C overnight. Secondary antibody incubation occurred for 1 hour at room temperature at a 1:5000 dilution. Proteins were visualized by enhanced chemiluminescence (Pierce).

3.2.8. MTT Assay

HeLa cells (100K) were plated on 35-mm confocal dishes (MatTek). After 24h, cells were transiently transfected as shown using Lipofectamine LTX (Invitrogen) according to manufacturer's instruction. After 24h growth in full medium, cells were serum starved (DMEM + 0.1% BSA) for 5h followed by treatment with vehicle or PMA as shown. Cells were washed once with PBS and fixed with fresh 4% paraformaldehyde for 10 min. After 3 x PBS washes, cells were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes, washed with PBS and blocked with 2% human serum for 1h at room temperature. After incubation with primary antibodies (in 2% serum, overnight at 4°C), cells were incubated with oligonucleotide labeled secondary antibodies. The PLA assay was performed according to manufacturer's protocol (Olink Bioscience, Uppsala, Sweden) The signal from each detected pair of PLA probes, visualized as an individual fluorescent spot, was acquired with a Leica TCS SP8 microscope. PLA signals were counted and quantified as shown.

3.2.9. Immunoprecipitation

HEK cells were co-transfected with PLD1 and PKC-expressing plasmids and treated with PMA as described above. After washing with 1X PBS, cells were collected and lysed in lysis Buffer (50 mM Tris-HCl, ph 7.4, 150 mM NaCl, 1mM EDTA, and 1% Triton X-100) with protease and phosphatase inhibitor cocktails (Sigma Aldrich) on ice for 20 min. Lysates were cleared by centrifugation (12000 rpm, 10 min, 4C) and protein concentration was determined by Bicinchoninic Acid Assay (BCA). Lysates containing 500μg - 1mg protein were pre-cleared with protein A/G agarose beads (Santa Cruz) for 1h at 4C and subsequent immunoprecipitations were

carried out using anti-GFP antibody by end-to-end rotation overnight at 4C. The next day, protein A/G agarose beads were added to the samples and incubated for another 1h at 4C. Antigen-antibody-agarose complexes were separated by centrifugation and washed three times with lysis buffer and three times with 1X TBS. After boiling with sample loading buffer, proteins were resolved on 4-20% TGX gels (BioRad Laboratories) and immunoblotting was carried out as described above.

3.2.10. Transwell Assays

Migration was assessed by transwell assay using FluoroBlock 8μm HTS Cell Culture Inserts for 24-well plates (BD Biosciences) as described previously (23) with minor modifications. Cells were not starved prior to being placed in the transwell upper chamber, and were stained with BD calcein AM fluoresent dye for 1 hour prior to reading in Spectramax M5 plate reader at 540nm.

3.2.11. Statistics

Statistical significance was calculated with student's t-test or by two-way ANOVA with Bonferroni Post test where appropriate. A p-level of below 0.05 was considered to be statistically significant.

3.3. Results

3.3.1. Activation of mTORC1 in response to sustained PKC activation requires endocytosis and PLD activity.

Acute activation of cPKCs results in their translocation to the PM. In contrast, sustained activation of cPKCs with PMA or sustained activation of membrane receptors results in their internalization from the PM to the PCN, a perinuclear PKC- and PLD-dependent and containing

subset of recycling endosomes(8,9,31). We recently reported that several of cPKC substrates in HEK cells were phosphorylated with delayed kinetics that better match PKC internalization and translocation to the PCN rather than acute PKC translocation to the PM. The results also identified p70 S6K, a major substrate of mTORC1, as a putative substrate of sustained PKC activity (23). Given the central role of mTORC1 in regulating cell growth and proliferation in response to environmental cues, and that this pathway is often dysregulated in pathological conditions including cancer (83), it became important to define the role of sustained PKC activation and internalization in regulating the mTORC1 pathway. The internalization of PKC in response to PMA requires PLD activity and is prevented by inhibitors of endocytosis(9). Thus, to determine if PKC internalization is required for PMA activation of mTORC1, we investigated if PMA phosphorylation of S6K at Thr-389, a known mTORC1 phosphorylation site, met the above criteria. For these studies, hypertonic sucrose solution was utilized to inhibit clathrin-mediated endocytosis followed by stimulation with 100nM PMA for 60 min as shown (**Fig. 6A**). As seen before (23), PMA induced a strong increase in S6K phosphorylation in control cells. However, inhibition of clathrin-dependent endocytosis resulted in total inhibition of S6K phosphorylation compared to untreated control, thus suggesting that endocytosis is required for PMA activation of mTORC1.

To further evaluate the role of the PLD/endocytosis pathway, the effects of PLD inhibition on mTORC1 activation were investigated. In our previous study (23), we utilized 1-butanol to implicate PLD in regulation of mTORC1; however, butanol affects both PLD isoforms and we could also not rule out other non-specific effects. Accordingly, to further probe the role of PLD, we utilized two distinct inhibitors of higher specificity - EVJ for PLD1 and JWJ for PLD2 (84,85). Results showed that inhibition of PLD1 had a modest effect on S6K phosphorylation induced by PMA; in contrast, inhibition of PLD2 prevented PMA effects on S6K more robustly (**Fig. 6B**). To further specifically implicate PLDs, an siRNA approach was employed. As can be seen, PLD1 and PLD2 siRNA significantly reduced the levels of their respective isoforms; however, while knockdown of PLD1 had a minimal effect, PLD2 knockdown markedly reduced PMA-induced S6K phosphorylation (**Fig. 6C**). Moreover, comparable effects were also seen with 4E-BP1, a distinct substrate of mTORC1, with PLD2 siRNA strongly inhibiting phosphorylation of 4E-BP1 as seen by a reduction of 4E-BP1

bandshift (**Fig. 6C**). These results are consistent with the effects of PLD pharmacological inhibitors observed above, and taken together, suggest a major role for PLD2 in PMA regulation of mTORC1 (37).

Previous studies on regulation of PLD by PKC identified a crucial residue in PKCα (F663) that was necessary for interaction of PKCα with PLD and its subsequent activation, effects that were absent in an F663D mutant (86,87). Notably, PKCα and βII have some homology in this region with PKCβII possessing a similar phenyalanine residue at position 666. Thus, we speculated that an F666D mutant of PKCβII would similarly be unable to interact with PLD. To confirm this, we assessed two of the criteria previously established by Exton and coworkers (86,87). In the first criterion, PKCα-wild-type (WT) and PLD1 but not the FD mutant was able to translocate to the perinuclear region upon sustained PMA stimulation. In the second, PKCα-WT and PLD1 were found to co-immunoprecipitate following PMA treatment demonstrating a direct interaction, whereas the FD mutant does not.

Utilizing these two criteria, the effects of F666D mutation on PKCβII localization and interaction with PLD were assessed. As can be seen, sustained PMA treatment induced translocation of PKCβII-WT to the perinuclear region where it colocalized with both PLD1 and PLD2 (**Fig. 7A**). In contrast, PKCβII-F666D acutely translocates to the PM in response to PMA, but is unable to internalize upon sustained PMA treatment (23). Furthermore, neither PLD1 nor PLD2 were internalized to the perinuclear region, nor do they colocalize with PKCβII-FD following sustained PMA treatment (**Fig. 7A**). To consolidate these results, proximity ligation assays (PLA) were performed to evaluate the interaction of PLD1 with PKCβII-F666D or WT. In PLA assays, to achieve a signal, the two proteins that are probed for have to be within 40nm of each other (88). Following PLA analysis, signals are quantified as the number of positive 'spots' per cell. As can be seen, results indicated that PMA increased the proximity of PLD1 and PKCβII-WT but this was not observed with the F666D mutant (**Fig. 7B**). Finally, the direct interaction of PKCβII-FD or WT with PLD1 was assessed by immunoprecipitation. For this, cells were co-transfected with HA-tagged PLD1 and either GFP-tagged PKCβII-FD or WT. Cells were serum starved and stimulated with vehicle of PMA for 30 min, followed by an immunoprecipitation of PKC as described in Methods. Immunoprecipitates were probed for both

HA-PLD1 and GFP (**Fig. 7C**). As can be seen, there was a minimal interaction of either PKCβII with PLD1 in unstimulated conditions. Notably, PMA stimulation induced an increase in the level of PLD1 in immunoprecipitates from cells transfected with PKCβII-WT. Importantly, this was completely absent in cells transfected with PKCβII-F666D. Thus, PMA induces an interaction of PKCβII with PLD1 but this is completely abrogated by the F666D mutation. Taken together with the above results and those of the Exton group with PKC, this confirms that interaction of PLD with PKCβII is required for internalization of both enzymes in response to sustained PMA treatment and neither interaction, nor internalization, occurs with the PKCβII-F666D mutant.

Having confirmed that PLD1 and 2 are unable to translocate to the pericentrion in the presence of the F666D mutant of PKCβII, this mutant was utilized to determine if the effect of PKC on mTORC1 requires interaction of PKC and PLD. For this, HEK cells stably expressing vector, PKCβII-WT, or PKCβII-F666D were generated and levels of p-S6K were assessed (**Fig. 7D**). As can be seen, cells overexpressing PKCβII-WT displayed a significantly higher level of S6K phosphorylation compared to vector controls. In contrast, the levels were significantly blunted in PKCβII-F666D cells and were comparable to vector controls. These results demonstrate that interaction of PKC and PLD is required for S6K phosphorylation and, taken together with all the above results, this strongly suggests that internalization of PKC is necessary for mTORC1 activity in HEK cells.

3.3.2. Dysregulation of endogenous PKC-β expression drives proliferation and migration in a PLD- and mTOR-dependent manner.

Thus far, results suggest that mTORC1 activation requires sustained PKC signaling and show that overexpression of PKCβII-WT but not FD in HEK cells increases mTORC1 activity, as evidenced by increase phosphorylation of S6K and 4E-BP1. This suggests a role for increased PKC expression in driving the mTOR pathway; indeed, previous studies had also suggested a role for increased expression of PKC as one possible mechanism for sustained activation of PKC (89). Thus, we speculated that this mechanism might play a role in cases where PKC is

endogenously overexpressed. To explore this, we initially assessed endogenous PKCβII expression in a number of cancer cell lines. To this end, representative lines from different tissues such as lung (A459 and H157), breast (MDA-MB-231 and MCF-7), colon (murine CT26), lymphoma (U937) and cervix (HeLa) were selected. As reference markers, the stable PKCβII-WT overexpressing HEK cells and wild-type HEK cells were utilized, as the latter possess low endogenous expression (**Fig. 8**). Western blot analysis revealed low PKCβ expression in all cells examined except for A549 cells, where expression was higher than that seen in the stably expressing HEK cell lines. Accordingly, A549 cells were utilized for subsequent experiments.

Next, it became important to determine if the higher expression of PKC would drive the growth of A549. Initial examination of the growth of these cells by cell number and MTT assay (**Fig. 9A**) revealed significantly higher growth of A549 compared to HEK evident by 48 hours, and further enhanced by 72 hours. To determine if PKC activity plays an active role in the growth of A549 cells, two inhibitors were utilized – enzastaurin – a PKC-β inhibitor, and Go6976, an inhibitor of cPKCs that we have previously shown to inhibit internalization of PKC (37). The results indicated that both inhibitors had a small but significant effect on growth of HEK at 48 and 72 hours (**Fig. 9B**), reducing cell numbers by approx. 25 % over this time course. In contrast, both inhibitors reduced the proliferation of A549 cells by 60-70% with effects seen as early as 24 hours post seeding and extending up to 72 hours (**Fig. 9B**). Indeed, in the presence of either inhibitor, the growth of A549 cells became closer to that of HEK cells. To consolidate these observations in A549 cells, and to avoid off-target effects of inhibitors, an siRNA approach was utilized. A549 cells were transfected with negative control (AStar) or PKCβII siRNA and 72h later, cell number and PKCβII protein levels were assessed (**Fig. 9C**). As can be seen, siRNA knockdown of PKCβII significantly reduced A549 cell growth to approximately 50% of cells treated with control siRNA. Strikingly, the extent of growth inhibition seen with PKCβII siRNA correlated with the extent of PKCβII knockdown observed at the protein levels (**Fig. 9C, inset**). Taken together with the pharmacological data, this strongly suggests a driving role for PKCβII in A549 cell growth.

Thus, next it was necessary to determine if this role is PLD-dependent. The PLD inhibitor FIPI (51) caused a significant reduction in cell proliferation of A549 cells with no significant effect seen on HEK (**Fig. 9D**), and again with PLD inhibition, the growth of A549 became similar to that of HEK cells. Thus, the proliferation of A549 cells is both PKC- and PLD-dependent.

Given the growth advantage imparted by PKC in A549 cells, it became important to determine if this pathway also regulates other distinct oncogenic properties. Therefore, migration of A549 cells was evaluated compared to HEK cells using a transwell migration assay and the role of PKC and PLD in this process was determined. The results showed that A549 cells displayed a high capacity for migration that was noticeably greater than that of HEK cells. Importantly, inhibition of PKC with Go6976 or PLD with FIPI had no effect on migration of HEK cells but significantly reduced migration of A549 cells (**Fig. 9E**).

The effects of PKC and PLD inhibitors on proliferation and migration of A549 cells, as well as mTORC1 activation led us to speculate that the mTORC1 pathway was also contributory for these properties of A549 cells. Accordingly, the mTOR inhibitor rapamycin was utilized to assess this. Strikingly, the results indicated that rapamycin had modest effects on proliferation of HEK cells as assessed by the MTT assay (approx. 30%, **Fig. 10A**) but had no appreciable effects on cell number (**Fig. 10B**), nor had any effect on migration of HEK cells (**Fig. 10C**). In contrast, rapamycin significantly reduced A549 cell growth both with MTT assay and cell number (**Fig. 10A, B**) and strongly inhibited the migration of A549 cells (**Fig. 10C**).

Collectively, all these results suggest that the elevated expression of PKCβII in A549 cells results in enhanced mTORC1 activity, proliferation, and migration that are dependent on both the PLD and mTOR pathways. In contrast, the growth and migration of HEK cells with much lower PKC expression is largely independent of both PLD and mTOR. In order to evaluate for effects of differential expression of components of the mTOR pathway between HEK and A549 cells, the levels of both upstream (Akt, TSC2, mTOR) and downstream (S6K, 4E-BP1) components were compared between HEK and A549 cells (**Fig. 10D**). Strikingly, the levels of mTOR, S6K, TSC2, 4E-BP1 and Akt were all higher in HEK compared to A549. Furthermore, a notable upshift was observed in 4E-BP1 of A549 cells compared to HEK cells consistent with

higher "basal' mTORC1 activity. Taken together, these results suggest that the differential sensitivity of HEK and A549 cells to rapamycin is not due to higher levels of mTORC1 pathway components in A549 cells.

3.3.3. Overexpression of PKCβII is sufficient to confer oncogenic properties in a PLD- and mTOR-dependent manner.

The results thus far indicated that high PKCβII expression was necessary for the increased proliferation and migration of A549 cells (at least compared to HEK cells), and this was dependent on both PLD and mTOR. This raised the question whether overexpression of PKCβII would be sufficient to enhance proliferation and migration of a non-tumorigenic cell line. We also hypothesized that this would be mediated through the PLD and mTOR pathways. To explore this, HEK cell lines stably overexpressing PKC-WT were utilized compared to cells expressing an out-of-frame PKC as a control. Furthermore, to functionally implicate the PLD pathway, cells stably expressing the PKC-F666D mutant were used.

Analysis of cell growth showed that cells overexpressing PKCβII WT significantly increased proliferation compared to cells expressing out of frame PKC (vector control cells); indeed, by day three, the cell number of PKCβII WT cells was 40% higher (**Fig. 11A**). On the other hand, the growth of the PKCβII FD cells was not significantly different from controls, indicating that interaction of PKCβII with PLD is required for increased growth. Analysis of cell migration by transwell assay demonstrated that PKCβII-WT overexpression also significantly enhanced cell migration whereas PKCβII-FD overexpression had little effect (**Fig. 11B**), again suggesting dependence on the PKC-PLD interaction. To confirm that the PKC-induced changes in proliferation require enzymatic activity, the PKCβII inhibitor enzastaurin (90) was utilized. For this and subsequent experiments, a three day time point was utilized, as this showed the largest effect. Notably, enzastaurin had no significant effect on growth of control cells and a small but insignificant effect on the growth of PKCβII FD cells (**Fig. 11C**). In contrast, the proliferation of PKCβII WT cells was significantly reduced confirming that the observed effects on cell growth were through PKC activity (**Fig. 11C**).

The lack of effect of the PKCβII FD mutant on proliferation and migration suggested that PLD is required for the PKC-mediated effects. To further confirm this, PLD activity was blocked using the PLD inhibitor FIPI (51). As can be seen, highly similar to enzastaurin, FIPI reduced proliferation of PKC-WT cells to that of control cells, but with no significant effect on the growth of control and PKC-βII-FD cells (**Fig. 11D**). These results were further confirmed utilizing the MTT assay as an additional measure of cell growth and cell viability (**Fig. 11E**). Moreover, FIPI also significantly reduced the migration of PKC-βII WT cells to that of control, but had no significant effect on control cells or PKC-βII-FD expressing cells (**Fig. 11B**). Thus, both PLD activity and PKC-PLD interaction are required for PKC effects on proliferation and migration.

We next considered the hypothesis that PKC effects were also conferred in an mTOR-dependent manner and utilized rapamycin to explore this. Interestingly, rapamycin treatment had little to no effect on the growth (**Fig. 12A**) or migration (**Fig. 12B**) of either control or PKCβII-FD cells, but it significantly inhibited both properties of PKCβII-WT cells bringing them to levels comparable to that of control. Thus, PKC overexpression is sufficient to enhance oncogenic properties of non-tumorigenic HEK cells in a manner dependent on PLD activity, PKC-PLD interaction, and the mTOR pathway.

3.4. DISCUSSION

In this study, we have explored the role of cPKCs in regulation of mTORC1 and the biological importance in conditions of sustained PKC activity. The results demonstrate that increased PKC expression is both necessary and sufficient to confer oncogenic properties – namely proliferation and migration - on non-tumorigenic cells. Crucially, the effects of PKC were dependent on PLD, required PKC-PLD interaction, required PKC internalization, and were dependent on the mTOR pathway. Collectively, these findings establish a novel pro-oncogenic pathway involving PKC, PLD, and mTOR, and they also suggest a role for internalized PKC and the PCN as a novel compartment in the sustained action of cPKC in this process.

Functionally, results from this study demonstrate the existence of a coordinated pathway in oncogenesis involving cPKC, PLD and mTOR; indeed, previous studies have reported that PKC overexpression correlates with enhanced oncogenic activity (91). Other studies have demonstrated dysregulation of mTOR signaling in numerous cancers (92,93), and some studies have also implicated PLD in oncogenesis (94,95). Given the enhancement of S6K phosphorylation by PKC overexpression, we postulated that oncogenic signaling downstream of sustained PKC activity would require both PLD and mTOR. Utilizing both inhibitors and siRNA, results demonstrated that PKCβII was necessary for the enhanced proliferation and migration of A549 cells relative to non-cancerous HEK cells. Reciprocally, PKCβII overexpression was sufficient to impart enhanced proliferation and migration of non-tumorigenic HEK cells to a level resembling that of A549 cells. The results clearly showed that PKCβII effects on proliferation and migration were PLD dependent and, crucially, were also mediated through the mTOR/S6K pathway as evidenced by the increased sensitivity of oncogenic properties of A549 and HEK-PKCβII cells to rapamycin. Overall, these results identify the PLD and mTOR/S6K pathways as primary mediators of oncogenesis by cPKCs.

These results in turn raise therapeutic possibilities. Rapamycin is already in clinical trials in various cancers and 'second generation' inhibitors of mTOR are under development (96,97). Inhibitors of PKCßII are also in clinical trials (98,99). Thus, it would be of great interest to determine if the cPKC/PLD/mTOR pathway is a more highly coordinated pathway in some cancers. If so, and as supported by the results from this study, this raises important questions about the development of optimal strategies for inhibition of this pathway e.g. coordinated inhibition of multiple targets in the pathway in order to overcome resistance and/or inhibition of one component of this pathway in combination with targeting distinct pathways. Irrespective, the results suggest that increased PKC in cancer generates an "addiction" of these cells not just to PKC activity but also to mTOR and PLD.

The results presented herein also expand on the emerging roles of the PCN, a PKC- and PLD-dependent and containing subset of recycling endosomes formed in response to sustained PKC activation by PMA or GPCRs (8,9,31). Previous studies have demonstrated PCN-dependent cross desensitization of receptors (8) and have implicated the PCN in the delayed

phosphorylation of cPKC substrates (23). This latter role is further developed in this study utilizing S6K as a key downstream target. Indeed, results presented herein strongly implicate the PCN in regulating the mTOR pathway in response to PMA. Consistent with this, delayed S6K activation in response to epidermal growth factor was found to be PKC-dependent (22), and studies with the GPCR agonists serotonin and angiotensin II have also reported PKC-dependent activation of S6K (100). Notably, these stimuli also induce the formation of PCN (8), suggesting it may be a general mechanism by which sustained PKC activity regulates the mTOR pathway. Consistent with this hypothesis, both Rheb and mTOR were reported to localize within the endolysosomal system (101,102) and the Rab family of proteins, key regulators of cellular trafficking, can also regulate mTOR activity (103). Studies investigating the mechanisms by which the PCN regulates the mTORC1 pathway are currently ongoing in our laboratory.

Another mechanistic consideration relates to the role of PLD. As noted above, this work identified PLD as an intermediate in PKC-dependent regulation of S6K and oncogenesis. Importantly, previous studies have identified PLD as a regulator of the mTOR/S6K pathway in response to serum, growth factors and amino acids (104) and a variety of somewhat conflicting mechanisms by which PLD regulates mTOR have been proposed. These include direct regulation of the mTORC1 complex assembly, activation of ERK upstream of mTORC1, and displacement of mTOR inhibitors (105-107). Increased PLD expression was also reported to increase mTOR activity and induce rapamycin resistance (64). The current study now establishes PLD as a crucial regulator of PMA- and PKC-dependent mTOR activation. Strikingly, this renders cancer cells more sensitive to inhibitors of both PLD and mTOR. Our results raise the possibility that formation of the PCN is a hitherto unappreciated mechanism by which PLD may regulate mTOR/S6K signaling.

In conclusion, this study demonstrates a role for sustained activation of PKC in regulation of the mTOR/S6K pathway. We further show that increased PKC expression is both necessary and sufficient to confer oncogenic properties - enhanced proliferation and migration – and this is dependent on PLD activity, PKC-PLD interaction and the mTOR/S6K pathway. These results further suggest that inhibitors of PLD or mTOR would be beneficial in cancers where PKC overexpression is a contributing or driving factor.

Chapter 4

Protein Kinase C Regulation of Mammalian Target of Rapamycin

Abstract

The mammalian target of rapamycin (mTOR) signaling pathway integrates intracellular and extracellular signals and plays a key role in cell metabolism, growth, proliferation and survival (108-110). The mTOR protein interacts with two sets of proteins to form two distinct multi-protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 responds to growth factors, energy status, oxygen levels and amino acids and then transduce these signals to downstream actions, such as protein synthesis, autophagy, and lipid synthesis (111).

PKC has been shown to activate the mTORC1 signaling pathway through MEK/ERK (112). However a more recent study shows that the requirements for MEK/ERK in the control of mTORC1 vary between different cell types (113), which implicate additional signaling connections between PKC and mTORC1. Here, we provide evidence that sustained activation of PKC induces the accumulation of mTOR in the perinuclear lysosomes, which is close to the cPKC-containing endosomes, but the two compartments are distinguished. We demonstrate that the accumulation of mTOR requires PKCη. In addition, amino acids also utilized PKCη to activate mTORC1 and the translocation of mTOR to lysosomes. These data demonstrate that sustained activation of PKC activates mTORC1 through a novel and more complicated mechanism, which requires two distinct elements: a) the formation of the cPKC-containing endosomes, and b) the accumulation of mTOR in the perinuclear lysosomes in an nPKC-dependent manner.

4.1. Introduction

The mTOR signaling pathway is one of the most important signaling pathways in eukaryotes that serves as a regulator of the response to the change of nutrients (114), growth factors (111) , energy stress and oxygen (111). Its activation triggers downstream signals to regulate cell metabolism, growth, proliferation, and survival. mTOR interacts with different sets of proteins to form two distinct multi-protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). It is known that growth factors, such as insulin, activate mTORC1 through the PI3K/AKT signaling pathway, which suppresses tuberous sclerosis complex (TSC1/2) by phosphorylation on Ser939 and Thr1462 of TSC2(115). TSC1/2 acts as a GTPase- activating protein for the Ras-related small G protein Rheb, an important activator of mTORC1. Thus, TSC1/2 serves as a negative regulator of mTORC1 signaling (116). Energy stress, such as glucose deprivation and hypoxia can lead to down regulation of mTORC1 signaling by activating AMP-activated protein kinase (AMPK) (117) or through REDD1 (regulated in development and DNA damage responses 1) (118). It has been reported that activated AMPK can phosphorylate TSC1/2 to suppress mTORC1 and can directly phosphorylate the mTOR binding protein raptor on Ser722 and Ser792 to induce inhibition of mTORC1 (117). mTORC1 senses the change of amino acids through a different mechanism which does not require the regulation of TSC1/2. It has been proposed that amino acid availability induces translocation of mTOR to the surface of the lysosomes containing Rheb for mTOR activation in a Rag GTPase- dependent manner(101). More recent studies show that the novel Ragulator protein complex is required for localizing the Rag complex and mTORC1 to the lysosomal surface for mTOR activation (119). In addition, amino acid-induced mTOR activation also requires interaction of vacuolar H+ adenosine triphosphatase ATPase (v-ATPase) with Ragulator protein complex (120).

The protein kinase C (PKC) family consists of 11 isoforms, which are grouped into 3 subfamilies (classical, novel, and atypical) based on their structures and activators (33). The classical PKCs (cPKCs) are activated acutely in response to stimulation of phospholipases C (PLC) by receptors, such as angiotensin II type 1A receptor (35) and serotonin receptors (121). Activated PLC-β or PLC γ hydrolyze phosphatidylinositol 4,5 bisphosphate and releases inositol

1,4,5- triphosphate (IP3) which releases intracellular calcium and diacylglycerol (DAG) which directly activates cPKCs and facilitates translocation of cPKCs from the cytosol to the plasma membrane within 60 seconds (36).

In addition to the above well-established paradigm of acute activation and translocation of cPKCs, our group previously reported that sustained activation of cPKCs by PMA or activation of some G-protein-coupled receptors (e.g. serotonin receptor) resulted in translocation of cPKCs, PLD, lipids and some receptors (e.g. serotonin receptor, epidermal growth factor receptor) to the pericentrion, a dynamic recycling endosomes formed by the action of PKC (8,31). Further studies indicate that the formation of the pericentrion required sustained activities of cPKCs and PLD and is dependent on clathrin-mediated endocytosis (37). Moreover, with sustained activation of cPKC, the cPKC-containing endosomes relocate close to Rab11 endosomes. (9). Phosphatidylcholine- specific phospholipase D (PC-PLD) is one of the major targets of PKC (122). Activated PC-PLD hydrolyzes phosphatidylcholine to generate choline and phosphatidic acid (PA). Some studies have shown that PLD-generated PA was able to activate mTOR (123,124) and a more recent study indicates that PA competes with the mTORC1 inhibitor, FK504 binding protein 38 (FKBP) 38 and allosterically induces the activation of mTORC1 (105).

It has been reported that PKCs are involved in the mTOR signaling pathway in various cell lines. For example in adult cardiac muscle cells, PKCε and PKC δ are required for ET-1 induced mTORC1 activation, and PKCδ is involved in insulin-stimulated activation of mTORC1(125). Interestingly, Fan et. al. showed that in glioma increased EGFR protein levels usually correlate with elevated activity of mTORC1 and PKC and that signals from EGFR to mTORC1 are through PKCα and independent of Akt (22). In addition, PKCξ, an atypical PKC, was implicated in abnormal mTOR regulation in follicular lymphoma cells in a MAPK-dependent manner (126). Previous studies showed that the phorbol ester PMA (phorbol 12-myristate 13-acetate), a well-known agonist of PKC, activates mTORC1 in a PKCs- dependent manner and involves ERKS and 90-kDa ribosomal S6 kinases (p90RSK) (112). However a more recent study indicates that p90RSK is actually not required for the activation of mTORC1 induced by PMA, and the requirement of MEK/ERK for the mTORC1 activation is variable

between different cell lines (113). So the mechanisms by which PKCs are involved in mTORC1 signaling are still unclear. In our recent research, we find that phosphorylation of p70S6K, one of major substrates of mTORC1, induced by sustained activation of PKC (30 min and longer), is different from the phosphorylation of Erk and Ezrin, which achieve a maximum of phosphorylation at 5 min. By studying the mechanism by which sustained activation of PKC induces the activation of mTORC1, we found that the activation of mTORC1 is rapamycin sensitive as expected, but more interesting it is cPKC-, PLD- and endocytosis- dependent (10).

In this study, we have further investigated the mechanism by which the PKC regulates the activation of mTORC1. We find very exciting results that the activation of mTOR stimulated by sustained activation of PKC involves a dual mechanism: 1) activation of nPKCs mediates the translocation of mTOR to the perinuclear lysosomes; 2) the formation of the cPKC-containing endosomes induced by sustained activation of cPKC facilitates the accumulation of PLD2 and PA in the PKC-dependent endosomes, which might trans-compartmently activates mTORC1 located in the perinulcar lysosomes.

4.2. MATERIALS AND METHODS

4.2.1. Materials

All cell lines were purchased from American Tissue Culture Collection (Manassas, VA, USA). Lipofectamine RNAiMAX were from Life Technologies (Grand Island, NY). X-tremeGENE 9 were from Roche (Indianapolis, IN). PMA, Gö6976, Bisindolylmaleimide I and U0126 were from EMD Millipore (Billerica, MA). Anti-phospho-p70S6K (Thr389), S6K, mTOR, phospho- Erk1/2 (Thr202/Tyr204), Erk1/2, EGFR, HA antibodies were from Cell Signaling Technology (Danvers, MA) and anti-Actin antibodies were purchased from Sigma Aldrich (St. Louis, MO). Anti-Lamp1, Lamp2, PKCδ and PKCε antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-EEA1 antibody was from BD Biosciences (San Jose, CA). siRNA for PKCη，PKCδ，PKCε，RagB, Ragulator 1 and Ragulator 3 were from Qiagen (Valencia, CA). The inhibitors specific for PLD2 (VU0364739(JWJ)) were a kind gift from Dr. Alex Brown (Vanderbilt University, TN).

4.2.2. Cell Culture

HEK293 cells were maintained in MEM medium supplemented with 10% (V/V) fetal bovine serum. Hela cells were grown in DMEM medium supplemented with 10% (V/V) fetal bovine serum. All cells were grown in a 5% CO2 incubator at 37°C.

4.2.3. Plasmid Construction

All plasmids were made by standard protocols. HA-tagged PLD2 and GFP-Spo20 were gifts from Dr. Michael Frohman (Stony Brook University, Stony Brook, NY). The wild type pBK-CMV-GFP-PKCα and PKCβII-mCherry were described previously (3,127). HA-tagged PKCη was a gift from Bernard Weinstein (Addgene plasmid # 21244) and HA-tagged FKBP38 was a gift from Yu Jiang (University of Pittsburgh, Pittsburgh, PA).

4.2.4. siRNA Transfection

siRNAs were transfected into HEK cells with Lipofectamine RNAiMAX respectively following the manufacturer's recommendation. Transfected cells were grown in medium with 10%FBS for 48 hours then followed with treatment.

4.2.5. Transient Transfection, Indirect Immunofluorescence, and Confocal Microscopy

Cells were plated on 35-mm confocal dishes (MatTek) at a density 3 to 5 $\times 10^5$ cells/dish. After 24 hours, X-tremeGENE 9 was used for transient transfection following the manufacturer's recommendation. After transfection cells were grown in normal medium with 10% FBS for 24 to 48 hours and then starved with medium without serum for 5 hours, followed by treatment. Cells transfected with green fluorescence protein (GFP) were fixed and examined as described before. For indirect immunofluorescence, the protocol was the same as described before. All images

were taken by Leica TCS SP8 and pictures are representative of three fields examined from three independent experiments.

4.2.6. Proximity Ligation Assay

Hela cells were plated on 35-mm confocal dishes (MatTek) at a density 3 to 5 ×104 cells/dish. After 24 to 48 hours, cells were starved for 5 hours followed by treatment with vehicle or PMA as shown. Cells were then fixed and permeabilized as described before. After incubation with primary antibodies (Lamp1 and mTOR), the proximity ligation assay were performed as described before.

4.2.7. Immunoprecipitation

HEK cells were transfected with FKBP38-expressing plasmid and treated with PMA as described above. After washing with 1X PBS, cells were collected and lysed in lysis Buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1mM EDTA, 1% Triton X-100 with 1X protease and phosphatase inhibitor cocktails. (Sigma Aldrich). Lysates (500 μg) were incubated with anti-HA antibody by end-to-end rotation overnight at 4C. After 24 hours, protein A/G agarose beads were added to the samples and incubated for 1 hour at 4C. Immunoprecipitates were collected by centrifugation and washed three times with lysis buffer and one time with 1X TBS. After boiling samples for 2-3 minutes, 20ul aliquots were analyzed by 4-20% TGX gels (BioRad Laboratories) and immunoblotting were carried out as described before.

4.2.8. Amino Acid Starvation and Recovery

Hela cells were plated on six-well plates at a density 3 to 5 ×104 cells/well. After 24 to 48 hours, cells were briefly washed with Hanks Balanced Salt Solution (Thermo Fisher Scientific) and then incubated with 2 ml of fresh Hanks Balanced Salt Solution for 45 min at 37 °C followed with addition of 2 ml DMEM with extra 1X amino acid (Thermo Fisher Scientific).

4.2.9. Statistic Analysis

Statistical significance was calculated with student's t-test or by two-way ANOVA with Bonferroni Post test where appropriate. A p-level below 0.05 was considered to be statistically significantly.

4.3. RESULTS

4.3.1. PMA induces the accumulation of mTOR in the perinuclear lysosomes, which is close to but distinct from the cPKC-containing endosomes.

In our previous study, we reported that with sustained activation of PKC p70S6K, a major substrate of mTORC1, was phosphorylated with delayed kinetics (23). The phosphorylation of S6K in response to sustained PKC activation requires PLD2 and endocytosis. Given the important role of mTORC1 in regulating cell growth, proliferation and metabolism and that this pathway is often dysregulated in human disease, including cancer, obesity, type 2 diabetes and neurodegeneration (93), it became of great importance to define the mechanism by which PKC regulates the mTORC1 pathway. Thus, to confirm the kinetics of phosphorylation of S6K by sustained activation of PKC, more time points of PMA treatment were investigated. HEK cells were treated with PMA for 5, 10, 15, 20, 30, 60 and 120 min, and phosphorylation status of p70S6K was analyzed by Western blotting. As shown in **Figure 13A**, the phosphorylation of p70S6K started after 15 min and achieved maximum at 60 min, which was consistent with the observation reported earlier (23). As previous studies showed that PMA activates mTORC1 in a PKCs- dependent manner and requires MEK/ERK and p90RSK (112). However, the mechanism remains unclear, and a more recent study indicates that p90RSK is not required, and the requirement of MEK/ERK for the activation of mTORC1 is variable among different cell lines (113). Therefore, we pretreated cells with MEK inhibitor U0126 and then followed with PMA treatment. Results showed that at a concentration of 0.5 μM U0126 was able to inhibit the phosphorylation of ERK more than 50%, but the inhibitor did not have any effect

on the phosphorylation of S6K induced by sustained treatment with PMA, thus suggesting that MEK/ERK is not required for the activation of mTORC1 induced by sustained stimulation of PKC (**Supplementary Fig. 1**).

Since the lysosomal localization of mTOR is essential for the activation of mTORC1 induced by amino acid, we examined the localization of mTOR upon sustained treatment with PMA. Interestingly, we found that with prolonged PMA treatment, more mTOR co-localized with Lamp1 in the perinuclear region, suggesting increased interaction of mTOR and Lamp1 and accumulation of mTOR in the perinuclear lysosomes (**Fig. 13B**). To consolidate these results, proximity ligation assays (PLA) were preformed to evaluate the interaction of mTOR with Lamp1. As can be seen, the PLA results indicated that PMA increased the proximity of mTOR and Lamp1 compared to the vehicle treatment (**Fig. 13C**).

As we previously reported that sustained stimulation of PKC with PMA led to sequestration of recycling endosomes and classical PKCs into a Rab11-positive juxtanuclear compartment, here we also examined the localization of PKCα after the treatment with PMA. As expected, with the prolonged PMA treatment, the overexpressed PKCα-GFP translocated from the cytoplasm to a perinuclear region and co-localized with Rab11-positive endosomes (**Fig. 13B**). Since both PKCα and mTOR translocated to perinuclear region upon PMA treatment, it is reasonable to speculate that if they are in the same region and/or in the same compartment. To explore this, we overexpressed PKCα-GFP in HEK cells and then checked the localization of PKCα-GFP and mTOR/Lamp1 upon PMA treatment. Results showed that PKCα-GFP was partially colocalized with mTOR and Lamp1 upon PMA treatment, suggesting that the mTOR containing lysosomes and cPKC-containing endosomes may be the same compartment (**Fig. 13D**). To further investigate this, we utilized cPKC, PLD and endocytosis inhibitors, as these inhibitors have been shown to inhibit the formation of the cPKC-containing endosomes. As can be seen, the cPKC inhibitor (Go6976), PLD inhibitor (1-Butanol) and endocytosis inhibitor (hypertonic sucrose) had no effect on the translocation of mTOR induced by PMA (**Fig 13E, F, G**). In addition, these inhibitors have been shown to inhibit the activation of mTORC1 with sustained stimulation of PKC, which implicated the important role of the cPKC-containing endosomes in the activation of mTORC1.

Collectively, all these results imply that upon sustained stimulation of PKC, mTOR accumulates in the perinuclear lysosomes coincident with the formation of cPKC-containing endosomes, which is different from the perinuclear lysosomes but is required for the activation of mTORC1.

4.3.2. Upon sustained stimulation of PKC, PA accumulates in the cPKC-containing endosomes activates mTORC1 through displacing the endogenous inhibitor FKBP38 from mTOR

Thus far, results suggest that the formation of the cPKC-containing endosomes is required for the activation of mTORC1. Since the mTOR-containing lysosomes and the cPKC-containing endosomes are localized in the perinuclear region and are close to each other, we speculated that the cPKC-containing endosomes activate the mTOR in the lysosomes through trans-compartment signaling. We previously reported that upon PMA treatment both PKCβII and PLD, which is the enzyme that catalyzes the hydrolysis of phosphatidylcholine to form PA, sequestrated into the cPKC-containing endosomes and only PLD2 but not PLD1 plays a major role in PMA regulation of mTORC1. Since PA has been shown to directly bind with mTOR through displacing its endogenous inhibitor FKBP38, it is rational to assume that PA generated by PLD2 accumulates in the cPKC- and PLD2- containing endosomes activates mTOR in the lysosomes. To further confirm the localization of PLD2 upon PMA, we cotransfected HEK cells with PKCα- and PLD2- containing plasmids or PKCβII- and PLD2- containing plasmids followed with PMA. Indeed, with sustained PMA treatment PLD2 sequestrated into the perinuclear region and colocalized with PKCα and PKCβII (**Fig. 14A**). Moreover, the sequestration of PLD2 was inhibited by pretreatment with cPKC inhibitor, Go6976, suggesting that the sequestration of PLD2 into cPKC-containing endosomes is cPKC-dependent (**Fig. 14A**).

Next, we analyzed the production of PA utilizing a fluorescent PA sensor consisting of enhanced GFP fused to a 40- amino acid PA-binding domain from the yeast Spo20 protein (51). We cotransfected HEK cells with the Spo20-GFP and PKCβII-mcherry followed with the prolonged PMA treatment. As shown in the **Fig. 14B**, in resting cells, the majority of the sensor

localized to the nucleus, but in cells treated with PMA, the sensor translocated in part to the plasma membrane and to the perinuclear region, which is where PKCβII resides. These data suggest that sustained stimulation of PKC induces the accumulation of PA in the cPKC-endosomes. As some studies have reported that PA could displace FKBP38 from mTOR and stimulates the activity of mTORC1 (105), we accessed the direct interaction between mTOR and FKBP38 upon PMA by immunoprecipitation. For this, HEK cells were transfected with HA-tagged FKBP38 and then treated with vehicle or PMA for 60 min followed by immunoprecipitation as described in the Methods. As can be seen, there was strong interaction of FKBP38 with mTOR in unstimulated condition (**Fig. 14C**). Notably, PMA stimulation induced a decrease in the level of mTOR in the immunoprecipitates (**Fig. 14C**). Taken together with the above results, this implicates that sustained stimulation of PKC induces the accumulation of PA in the cPKC- and PLD2- containing endosomes, which in turn activates mTOR through displacing the endogenous inhibitor FKBP38 from mTOR. However, we need to further verify the localization of PA (see chapter 6).

4.3.3. Novel PKC is required for mTORC1 activation by regulating mTOR localization

Since we observed the accumulation of mTOR in the perinuclear lysosomes upon PMA and some studies have reported that with amino acid stimulation, mTOR translocated to the surface of Rheb-containing lysosomes for the activation of mTORC1 in a RagA/B GTPase-, Ragulator- and vacuolar H+ ATPase- dependent manner (101,119), it is interesting to study the mechanism by which PMA induces the accumulation of mTOR in the perinuclear lysosomes. As shown above, the cPKC inhibitor, Go6976 had not effect on the translocation of mTOR upon PMA, and therefore here we examined the effect of PKC inhibitor, Bis on the localization of mTOR with PMA treatment. As can be seen, the pretreatment with Bis abolished the phosphorylation of p70S6K induced by PMA, and moreover inhibited the accumulation of mTOR in the lysosomes upon PMA (**Fig. 15A**). To further confirm the role of novel PKC in the regulation of mTOR translocation, we utilized the recently reported novel PKC activator, 10-Methyl-aplog-1 (128), which has been shown to preferentially activate novel PKC than classical PKC. Surprisingly, the nPKC activator induced the phosphorylation of p70S6K, and moreover

prompted the accumulation of mTOR in the perinuclear lysosomes (**Fig. 15B**). However, we still do not know if the nPKC activator also activates cPKCs and if Go6976 and JWJ inhibit the activation and the translocation of mTORC1. These results indicate that the translocation of mTOR induced by PMA may require the activity of novel PKC.

To further define which novel PKC isoform involves in the process, an siRNA approach was employed. As can be seen, PKCη, PKC**δ** and PKCε siRNA significantly reduced the levels of their respective isoforms (**Supplementary Fig. 2)**; however, while knockdown of PKC**δ** and PKCε had a minimal effect (**Supplementary Fig. 2)**, PKCη knockdown markedly reduced phosphorylation of p70S6K induced by PMA (**Fig. 15C**). Thus, next we examined the effect of knock down of PKCη on PMA-induced mTOR translocation. In the resting cells, mTOR was dispersed throughout the cells, but in cells with PMA stimulation, mTOR accumulated in the perinuclear lysosomes (**Fig. 15D**). However, in cells transfected with PKCη siRNA, treatment with PMA did not induce the accumulation of mTOR (**Fig. 15D**). Taken together, these data suggest that PKCη plays a major role in the accumulation of mTOR induced by PMA.

4.3.4. Amino acid activates mTORC1 in a PKCη- depdent manner

Thus far, results suggest that PKCη may play important role in regulation of mTOR localization upon stimulation. Our previous study reported that with sustained stimulation of PKC, vacuolar H+ ATPase, which is required for amino acid to promote mTORC1 translocation, translocated to a perinuclear region, suggesting PKC may regulate vacuolar H+ ATPase activity (23). Therefore, we speculated that PKCη is required for amino acids to regulate mTORC1 activity. To define the role of PKC in amino acid regulation of mTORC1, we used cPKC inhibitor (Go6976) and nPKC inhibitor (Bis). As shown in **Fig. 16A**, Go6976 had no effect on the phosphorylation of p70S6K induced by amino acid. In contrast, treatment with Bis significantly inhibited the p70S6K phosphorylation. Next, we knocked down PKCη using siRNA and found that knockdown of PKCη abolished the p70S6K phosphorylation prompted by amino acid (**Fig. 16B**). Moreover, in cells transfected with control siRNA, amino acids were able to promote mTOR translocation as expected, but in cells transfected with PKCη siRNA, amino acid

failed to do so (**Fig. 16B**). Together, these results implicate that PKCη is necessary for amino acids to activate mTORC1, possibly through regulating mTORC1 localization.

4.4. Discussion

In this study, we investigated the mechanism by which PKC regulates mTORC1. The results demonstrate that the activation of mTOR1 by sustained activation of PKC requires the formation of cPKC- containing endosomes and the accumulation of mTOR in the perinuclear lysosomes. Importantly, the translocation of mTOR required PKCη and meanwhile amino acids also utilized PKCη for the activation and translocation of mTOR. In addition, with sustained activation of PKC, PA also accumulated in the cPKC- containing endosomes, which is in close proximity to the mTOR- containing lysosomes, presumably activating mTOR through displacing its endogenous inhibitor FKBP38. Collectively, these findings defined a novel mechanism by which PKC regulates mTORC1 activity involving the formation of cPKC-containing endosomes and PKCη, and they also suggest a role for the novel localization of PA in the regulation, which directly activates mTOR trans-compartmentally.

It is the first time that PKCη is shown to be involved in the regulation of the translocation of mTOR. Our previous study reported that with sustained activation of PKC, V-ATPase accumulated in a perinuclear region (23). In addition, many other studies showed that PKC is involved in regulation of V-ATPase (129,130). However the specific mechanism by which PKC regulates V-ATPase and the exact localization of V-ATPase in cells with sustained activation of PKC are still not clear. V-ATPase has been shown to interact with Ragulator and promote mTORC1 translocation in response to amino acids stimulation (120). Thus, it is possible that the activation of PKCη induces the translocation of mTORC1 through V-ATPase. Interesting, it seems that the activation and translocation of mTORC1 upon amino acid stimulation also requires PKCη. However, the mechanism by which amino acid activates PKCη remains to be explored.

Another interesting finding refers to the accumulation of PA in the cPKC- containing endosomes. As reported above, with sustained activation of PKC, the PA sensor Spo-GFP

accumulated in the cPKC- containing endosomes, where PLD2 localized too. The cPKC-containing endosomes is in close proximity to the mTOR- containing lysosomes. Previous studies have defined both of PLD and PA as important players in the regulation of mTORC1 activity in response to serum, growth factors and amino acids (123,131). In addition, sustained activation of PKC reduced the interaction of mTOR and its endogenous inhibitor FKBP38, suggesting that PA induced by activation of PKC might be involved in the process. Thus, our results raise the possibility that the accumulation of PA induced by activation of PKC trans-compartmentally activates the mTOR in the lysosomes.

In conclusion, this study defines a novel mechanism by which PKC regulates mTORC1 activity and localization. In addition to the involvement of PKCη in regulating mTOR localization, the accumulation of PA and PLD2 in the cPKC-containing endosomes and the trans-compartmentally signaling seem critical for the activation of mTORC1 in response to sustained activation of PKC.

Chapter 5

Classical PKC/mTORC1 Signaling Pathway in Non-Small Cell Lung Cancer with EGFR Mutation

Abstract

Mutational activation of the epidermal growth factor receptor (EGFR) is a major player in the pathogenesis of non-small cell lung cancer (NSCLC) (132). NSCLC patients with constitutively active EGFR mutations respond well to EGFR tyrosine- kinase inhibitors (TKIs) initially; however, the majority of the patients eventually develop resistance to TKIs (133). As protein kinase Cα has important roles in cell regulation and proliferation and has been shown to play an important role in the development of drug-resistance, in this chapter we analyzed the role of PKCα in the regulation of the signaling pathways downstream of mutant EGFR. We found that NSCLC cell lines with constitutive active EGFR mutation tend to have high or moderate PKCα level. In addition, in HCC827 and H4006 cells which have an EGFR deletion mutation in exon 19, PKCα is always activated staying on the plasma membrane. In addition, the NSCLC cells are highly dependent on PKCα for the activation of mTORC1. We found that the activation of mTORC1 also requires the activity of PLD2, which have been shown to be important for the regulation of mTORC1 by sustained activation of PKC. Notably, inhibition of PKCα in HCC827 leads to caspase- 3- dependent apoptosis and significant decrease in cell survival in response to cellular stress induced by serum starvation. Our results identified important roles of PKCα in regulating mTORC1 activity in lung cancer cells, and present PKCα as a potential target of personalized treatment for NSCLC with constitutively active mutant forms of EGFR.

5.1 Introduction

Lung cancer is the leading cause of cancer- related deaths among both men and women over the world. The two major types of lung cancer are non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC can be further divided into three subtypes: squamous cell carcinoma, adenocarcinoma, and large-cell lung cancer. In NSCLC various mutations were identified and among them, exon 19 mutation characterized by in-frame deletion and exon 21 mutation resulting in L858R substitutions are major mutations (134).

It is reported that PKCα is highly expressed in NSCLC patients with higher level preferentially expressed in adenocarcinoma compared to squamous cell carcinoma(27). In addition, PKCα was shown to interact with discs large homology-1 to promote the migration of NSCLC cells(28). Notably, Chen's lab showed that down-regulation of PKCα by the miR-203 induces apoptosis in A549 cells and targeting PKCα by miR-203 plays significant role in cell proliferation, apoptosis and migration(29). All these data suggest that PKCα is an important target for treatment of lung cancer, but clinical trials using LY900003, and antisense inhibitor of PKCα in combination with anticancer drugs did not enhance survival in patients with advanced NSCLC(30). Thus, in order to better target PKCα as cancer therapy, more studies need to be done to explore the PKCα signaling in the NSCLC.

Since the mutational activation of the epidermal growth factor receptor (EGFR) is a major player in the pathogenesis of non-small cell lung cancer (NSCLC) (132). It is important to study the role of PKCα in the regulation of the signaling pathways downstream of EGFR mutation. In this chapter, we have investigated the PKCα status in NSCLC cells with constitutively active EGFR mutation and its role in the regulation of mTORC1 in response to the EGFR mutant. We found that NSCLC cell lines with constitutive active EGFR mutation tend to have high or moderate PKCα level. Notably, the NSCLC cells are highly dependent on PKCα for the activation of mTORC1. Thus defining a novel pathway for mTOR regulation in NSCLC.

5.2 Materials and Methods

5.2.1. Material

Dulbecco's Modified Eagle Medium with high glucose (DMEM) was from Invitrogen. The HCC827 and H292 cell lines was purchased from American Type Culture Collection. H4006 cells was a gift from Dr. John Haley (Stony Brook University, Stony Brook, NY). Enzastaurin and erlotinib were from LC Laboratories (Woburn, MA). Go6976 and U0126 was purchased from EMD Millipore (Billerica, MA). Anti-phospho-p70S6K (Thr389), S6K, mTOR, phospho- Erk1/2 (Thr202/Tyr204), Erk1/2, (E747-A750del Specific) EGFR, EGFR, P-Akt (Ser473), P-Akt (Thr308), Akt, PKCα and ErbB3 antibodies were from Cell Signaling Technology (Danvers, MA) and anti-Actin antibodies were purchased from Sigma Aldrich (St. Louis, MO). Anti-Lamp1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-EEA1 antibody was from BD Biosciences (San Jose, CA). siRNA for PKCα and PLD2 were from Qiagen (Valencia, CA). The inhibitors specific for PLD1 (VU0359595 (EVJ)) and PLD2 (VU0364739(JWJ)) were a kind gift from Dr. Alex Brown (Vanderbilt University, TN). All other reagents were from Sigma Aldrich (St. Louis, MO).

5.2.2. Cell Culture and Counting

HCC827, H4006 and H292 cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum. For cell counting, cells were trypsinized from culture dishes and 10μl of cell suspension was mixed with Trypan blue in a 1:1 ratio (v:v) for 1 minutes and then cell number was counted using the automated cell counter.

5.2.3. Plasmid constructs and transient transfection

All plasmids were made by standard protocols. Wild type GFP- PKCα was described previously. Cells were plated on 6- well plates at a density 5×10^4 cells/well. After 24 hours, X-tremeGENE 9 was used for transient transfection following the manufacturer's protocol. After transfection, cells were grown in normal medium with 10% FBS for 24 to 48 hours and then starved with medium without serum for 5 hours, followed by treatment.

5.2.4. siRNA Transfection

siRNAs were transfected into NSCLC cells with Lipofectamine RNAiMAX respectively following the manufacturer's recommendation. Transfected cells were grown in medium with 10%FBS for 48 to 72 hours then followed with treatment.

5.2.5. Immunofluorescence

Cells were plated on 35-mm confocal dishes (MatTek) at a density 3 to 5 $\times 10^5$ cells/dish. After 24 to 48 hours, cells were then starved with medium without serum for 5 hours, followed by treatment following with fixation and permeabilization as described previously. After incubation with 2% human serum for 1 hour, cells were stained with primary antibodies and secondary antibodies as described before. All images were taken by Leica TCS SP8 and pictures are representative of three fields examined from three independent experiments.

5.2.6. MTT Assay

5 x 10^4 cells were plated in 6- well plates and after 24 hours, cells were treated as indicated. 400µl MTT (Thiazolyl Blue Tetrazolium Bromide: 5mg/ml in PBS) and fresh medium was added to each well and following brief agitation, cells were incubated at 37C for 3-4 hours. Media was aspirated and 2ml MTT solvent was added. Following brief agitiation to ensure full dissolution, optical density was read at 595nm.

5.2.7. Immunoprecipitation

HCC827 were serum starved for 5 hours and then treated with vehicle, Gö6976 or erlotinib for 1 hour. After washing with ice cold PBS, cells were collected and lysed in lysis Buffer (50 mM Tris-HCl, ph 7.4, 150 mM NaCl, 1mM EDTA, and 1% Triton X-100) with

protease and phosphatase inhibitor cocktails (Sigma Aldrich) on ice for 20 min. Lysates were cleared by centrifugation and protein concentration was determined by Bicinchoninic Acid Assay (BCA). Lysates containing 500µg - 1mg protein were pre-cleared with protein G magnetic beads (EMD Millipore) for 1h at 4C and subsequent immunoprecipitations were carried out using anti-(E747-A750del Specific) EGFR antibody by end-to-end rotation overnight at 4 following the manufacturer's protocol. The next day, the pre-formed antibody-antigen complex was added to the protein G magnetic beads and incubated for another 1h at 4C. Antigen-antibody-bead complexes were separated by using the magnetic stand and washed three times with 1X PBS containing 0.1% Tween. After boiling with sample loading buffer, proteins were resolved on 4-20% TGX gels (BioRad Laboratories) and immunoblotting was carried out as described above.

5.2.8. Statistics

Statistical significance was calculated with student's t-test or by two-way ANOVA with Bonferroni Post test where appropriate. A p-level of below 0.05 was considered to be statistically significant.

5.3. Results

5.3.1. PKCα status in NSCLC

It was reported that PKCα is highly expressed in NSCLC and preferentially expressed in adenocarcinoma compared with squamous carcinoma of the lung. We analyzed the expression of PKCα by examining the Affymetrix U133-2 array data. As can be seen, PKCα (but not PKCβ) shows high or moderate expression in NSCLC cell lines with EGFR mutation and low PKCα is not seen in any NSCLC lines with EGFR mutation (**Fig. 17A**). To confirm the data is accurate, we randomly checked the protein level and mRNA level of PKCα in some NSCLC cells analyzed in the array. Indeed, in H292 cell line, which does not have EGFR mutation, we did not observe any protein level of PKCα and the mRNA level was also quite low; however, both

protein and mRNA levels of PKCα were significantly high in HCC827 harboring E746- A750 deletion in exon 19 of EGFR (**Supplementary Fig. 3**). We also observed high protein level of PKCα in H4006, a NSCLC cell line also containing the deletion mutation in exon 19 of EGFR (data not shown here). To evaluate the activity of PKCα in HCC827 and H4006, we examined the localization of PKCα in the cells under serum starvation. As shown in **Fig. 17B**, in HCC827 and H4006, PKCα stayed on the plasma membrane indicating that PKCα is active in the cells even under serum starvation. As expected, in A549 that only have wild type EGFR, inactive PKCα was mostly cytoplasmic (**Fig. 17B**). Collectively, these results indicate that NSCLC cell lines harboring activating EGFR mutation tend to have high expression of PKCα, and moreover PKCα in those cell lines is constitutively active, presumably stimulated by the EGFR mutant.

To confirm that the EGFR mutant induces the sustained activation of PKC, we co-transfected HCC292 cells with wild type GFP- PKCα and empty vector or mtEGFR (containing E746- A750 deletion in exon 19). As expected, PKCα remaied in the cytoplasm in cells transfected with PKCα and vector, but PKCα mostly was found on the plasma membrane when co-transfected with mtEGFR (**Fig. 17C**). Next, we examined the effect of EGFR specific inhibitor, erlotinib on the localization of PKCα in HCC827 cells. As can be seen, the inhibitor was able to change the localization of PKCα from the plasma membrane to the cytoplasm at a concentration of 500nM for 1 hour (**Fig. 17D**). Taken together, these results above suggesting that mtEGFR constitutively activates PKCα and drives the PKC isozyme to the plasma membrane.

5.3.2. Activation of mTORC1 in NSCLC cell lines with mtEGFR requires PKCα and PLD2

Since the data above suggest that mtEGFR consistently activates PKCα in HCC827 cells, it becomes important to determine the consequences of the sustained activation of PKCα in the NSCLC cell line. In chapter 3, we discussed the involvement of classical PKC in the regulation of mTORC1, so it is interesting to study the mTORC1 signaling in the NSCLC cell lines with mtEGFR. First, we assessed the phosphorylation level of p70S6K in both H292 and HCC827 cells. As can be seen, with serum starvation, the phosphorylation of p70S6K was much higher in

HCC827 cells compared with H292 cells (**Fig. 18A**). Interestingly, HCC827 cells did not respond to EGF stimulation suggesting that mTORC1 is already fully activated under serum starvation, probably through the mtEGFR/ PKCα signaling pathway (**Fig. 18A**). To verify that, we utilized classical PKC inhibitors, Go6976 and Enzastaurin. As can been seen, at concentrations as low as 50nM, Go6976 was able to inhibit the phosphorylation of p70S6K in HCC827 cells (Fig. 2B). Moreover, the more specific classical PKC inhibitor Enzastaurin also inhibited the phosphorylation of p70S6K (**Fig. 18B**). We likewise observed the similar inhibitory effect of these inhibitors in H4006 (**Supplementary Fig. 4**). To consolidate these observations in HCC827, and to avoid off-target effects of inhibitor, siRNA and CRISPR approaches were utilized. As can be seen, phosphorylation of p70S6K in HCC827 cells transfected with PKCα siRNA was reduced to approximately 20% of cells transfected with control siRNA. Similar results were observed in HCC827 cells in which PKCα was knocked out via CRISPR (**Fig. 18B**).

As we previously reported that the stimulation of mTORC1 in response to sustained PKC activation requires PLD activity, next we examined the role of PLD in the activation of mTORC1 in HCC827 cells. Surprisingly, HCC827 cells have much higher protein level of PLD2 compared with H292 cells but both cell lines have low protein levels of PLD1 (**Fig. 18C**). Moreover, the PLD2 inhibitor, JWJ but not PLD1 inhibitor, EVJ repressed the phosphorylation of p70S6K, suggesting that PLD2 is the major player in the activation of mTORC1 in response to sustained activation of PKC in HCC827 cells (**Fig 18C**). In addition, by analyzing the response of H4006 to different concentration of JWJ, we observed the similar inhibitory effect of JWJ on the phosphorylation of p70S6K (data not shown here).

In addition, to further confirm that the activation of mTOR is regulated by the mtEGFR/ PKCα pathway, we utilized PLCγ specific inhibitor, U73122 (135). We observed that treatment with U73122 induced the translocation of PKCα from the plasma membrane back to the cytoplasm and moreover, at a concentration of 10uM, the inhibitor effectively reduced the phosphorylation of p70S6K (**Fig. 18D**). These data implicate that the activation of mTORC1 requires the signaling from the mtEGFR transduced by PKCα and PLD2. Taken together with all the above results, this strongly suggests that PKCα and PLD2 are necessary for mTORC1 activity in NSCLC cells with mtEGFR.

5.3.3. PKCα is involved in the activation of PI3K/Akt induced by mtEGFR in HCC827

In addition to the involvement of PKCα in the activation of mTORC1, we also observed that classical PKC inhibitors, Go6976 and Enzastaurin, reduced the phosphorylation of Akt in HCC827 cells (**Fig. 19A**). We also found that knockdown PKCα via siRNA or CRISPR significantly repressed the phosphorylation of Akt (**Fig. 19A**). It has been shown that the Gab adaptor proteins play a role in activation of PI3K pathway by mtEGFR in EGFR- mutant NSCLC cell lines (136). Thus, to further evaluate the role of PKCα in the activation of PI3K/AKT by mtEGFR, we assessed the effect of Go6976 on the interaction of mtEGFR and Gab1. Notably, Go6976 and erlotinib both significantly decreased the interaction of Gab1 and mtEGFR (**Fig. 19B**). Together, these data implicate important roles of PKCα in the activation of PI3K by mtEGFR.

5.3.4. PKCα regulates cell survival and apoptosis in HCC827

Since our results strongly suggest that PKCα activates PLD and mTORC1 in response to mtEGFR, next we investigated oncogenic properties mediated by mtEGFR/PKCα/mTORC1 signaling pathway. We found that cancer cells with mtEGFR grow much better in serum free conditions compared with H292 cells with wild type EGFR (**Fig. 20A**). The growth advantage of HCC827 was impeded after treatment with erlotinib, to the level seen with H292 (**Fig. 20B**), suggesting that this advantage is contributed by the mtEGFR and not other differences between the two cell lines. Next we assessed the effects of inhibition of cPKC. Interestingly, the inhibition of cPKC abolished specifically this advantage for mtEGFR, and the same effects were observed with inhibition of mTOR (**Fig. 20C**). We also found that inhibition of cPKC induced caspase-3- dependent apoptosis, as did inhibition of mtEGFR (**Fig. 20D**). Taken together, these results begin to demonstrate that mtEGFR/PKC signaling pathway plays important role in cell survival and apoptosis.

5.4. Discussion

In this chapter, we examined the status of PKC α and investigated its role in regulation of mTORC1 activity in NSCLC cell lines with constitutively activated EGFR mutation. The results demonstrate that NSCLC cell lines with mtEGFR tend to have high and moderate PKCα. In addition, we found that mtEGFR cooperates with PKCα to regulate mTORC1 and PI3K/AKT signaling pathways, which play important roles in cell survival and apoptosis.

It is well known that wild type EGFR activates mTORC1 through PI3K/Akt, which phosphorylates and inhibits mTOR suppressor, TSC1/2 complex (115,137). However, these results presented here identify a novel signaling pathway in which PKCα is placed in a central position in regulating mTORC1 activity (**Scheme 3**). The mtEGFR is constitutively activated, which in turn stimulates PKCα through PLCγ. The sustained activation of PKCα then induces the activation of mTORC1 through PLD2. However, the mechanism by which PKCα regulates the mTORC1 activity here shows differences from what we described in chapter 4. In chapter 4, we observed that with sustained stimulation, cPKC translocated into a Rab11 recycling endosomes with the accumulation of PA and PLD2 in the same compartment. In contrast, in HCC827 and H4006 we observed the majority of PKCα stays on the plasma membrane and did not notice any clustering of PKCα in perinuclear region. One explanation for that is the cPKC-containing endosomes is a dynamic compartment, which transports proteins between the plasma membrane and recycling endosome, and protects these proteins from degradation. Indeed, it has been shown that the mtEGFR undergo aberrant traffic into the recycling compartment which allows mtEGFR escape the lysosomal degradation (67). Overall, these results identify the PKCα and PLD as primary regulator of mTORC1 activity in response to mtEGFR.

In addition to mTORC1 signaling, we also observed that PKCα is involved in the regulation of PI3K/Akt signaling. Inhibition of PKCα using pharmacological compounds and knockdown of PKCα through siRNA and CRISPR both effectively blocked the phosphorylation of Akt in HCC827 cells. It has been reported that mtEGFR uses ErbB3 to couple to the PI3K/Akt pathway (138). We knocked down ErbB3 in HCC827 cells but did not observe any decrease in phosphorylation of p70S6K. (data not shown). Consistent with the observation made by Politi's lab (136), our results show that knockdown of Gab1 significantly reduced the phosphorylation of

p70S6K (data not shown). Notably, PKCα inhibitor, Go6976 inhibit the interaction of Gab1 and mtEGFR, suggesting that cPKC plays a role in the coupling of mtEGFR to Gab1. Taken together, these results demonstrate that the activation of PI3K/Akt induced by mtEGFR requires PKCα.

These results in turn raise therapeutic possibilities. As the mtEGFR constitutively activates the downstream molecules, without the ligand binding, NSCLC cells with mtEGFR are consequently addicted to the mtEGFR signaling pathway. Thus, NSCLC patients with mtEGFR show more response to EGFR tyrosine kinase inhibitors, and significantly prolong median progression free with the inhibitors treatment (139,140). However, almost all the patients with initial response to gefitinib or erlotinib ultimately develop resistant to the inhibitors (141). There are several identified mechanisms underlying the acquired resistance and almost 50% of the acquired resistance developed is related to the appearance of the secondary mutation (T790M) in the mtEGFR (142), which attenuates the binding efficacy of TKI to the mtEGFR (143). Since our results indicate that PKCα is the major mediator downstream of mtEGFR, regulating mTORC1 and PI3K/Akt signaling pathways, PKCα should be considered as a potential target to treat NSCLC, which already developed the secondary mutation (T790M) in mtEGFR.

In summary, our data indicate for the first time that PKCα is essential for mTORC1 and PI3K/Akt signaling pathway in NSCLC with mtEGFR. In addition, NSCLC cell lines with mtEGFR preferentially express high or moderate level of PKCα. Moreover, the signaling pathways mediated by mtEGFR/ PKCα play important roles in cell survival and apoptosis. Therefore, targeting PKCα could benefit the NSCLC patients who have already developed secondary mutation (T790M) in mtEGFR, thus resulting in improved survival rate of patients.

Chapter 6

Discussion and Future Directions

6.1. Introduction

The work presented in this dissertation makes significant progress in understanding the regulation and function of PKC and its role in the regulation of mTORC1 and cancer biology. We defined a novel mechanism by which PKC regulates mTORC1 activity and provide evidence that for the first time demonstrate the important role of PKCα in regulation of mTORC1 and PI3K/Akt signaling pathways in NSCLC with mtEGFR. Although much progress has been made, many questions and interesting possibilities remain to be elucidated and are outlined below.

6.2. Define the mechanism by which PKCη regulates the localization of mTOR

Studies from Sabatini's group demonstrate that V- ATPase is involved in the localization of mTOR through interacting with Ragulator, a scaffolding complex that anchor RagA/B GTPase to the lysosome (120,144). Our previous study shows that with sustained activation of PKC, V- ATPase accumulates in the pericentrion or a closely related compartment, but the specific mechanism for that is not clear (23). Also some other studies suggest that PKC has a stimulatory effect on vacuolar H+ ATPase (129,145). Therefore, it is important to investigate if PKCη regulate the localization of mTOR in a V- ATPase- dependent manner. We will test if the knockdown of vacuolar H+ ATPase affects the phosphorylation of p70S6K or the translocation of mTOR induced by PMA treatment via western blot and immunofluorescence respectively. If we could not get good knockdown with those siRNAs, we could try to use concanamycin A and bafilomycin A1, which are pharmaceutical inhibitors of vacuolar H+ ATPase. Next, we will examine whether knocking down PKCη affects the activity of V- ATPase (146).

In addition, we have preliminary data suggest that novel PKC may be involved in the regulation of SH3BP4, which negatively regulates the translocation of mTORC1 (147). It has been reported that SH3 domain-binding protein 4 (SH3BP4) binds to the inactive Rag GTPase complex to impede its interaction with mTOR, which inhibits the translocation of mTOR to lysosomes for its activation upon amino acid starvation (147). SH3BP4 contains an SH3 domain, a PXXP motif, a bipartite nuclear targeting signal, and a tyrosine phosphorylation site (148).

Although there is no study showing that nPKC can directly phosphorylate SH3BP4, after analyzing the sequence of SH3BP4, we find that there are some potential nPKC phosphorylation sites in the protein (Group-based Predication System, version 3.0). So it is possible that nPKC phosphorylates SH3BP4 and changes its molecular confirmation, thereby releasing Rag GTPase complex to facilitate mTOR translocation. In order to confirm the role of nPKC in the regulation of SH3BP4, we will study if nPKC activation affects the binding of SH3BP4 to RagB using pharmaceutical inhibitors and siRNA technology. Also we will study if nPKC can phosphorylate SH3BP4 by applying in vitro kinase study. If we could confirm that nPKC can phosphorylate SH3BP4, next we need to determine which phosphoryaltion site induced by nPKC in SH3BP4 is important for disrupting the interaction between SH3BP4 and RagA/B GTPase.

A more recent paper from Sabatini's group indicates that GATOR1/2, newly identified complexes, regulate mTORC1 localization to the lysosomal surface through interaction with Rag GTPases (149). So it is possible that nPKC could interact with GATOR1/2 to regulate the localization of mTOR and this is worth to investigate.

6.3. Confirm that if PA in the cPKC-containing endosomes activates mTORC1 on the surface of the lysosomes trans-compartmentally

As shown in chapter 4, our results suggest that sustained activation of PKC induces the PA sensor Spo-GFP accumulated in the cPKC- and PLD2- containing endosomes, which are in close proximity to the mTOR- containing lysosomes. However the confocal results are not quantified, and thus more experiments are needed to further verify the localization of PA. Since PA has been implicated to localize in endosomes (51,150), next we will utilize immunofluorescence to check if the PA sensor co-localize with endosome markers, such as EEA1, Rab11. In addition, subcellular fractionation and quantification should be utilized to further confirm the localization of the sensor. Previous studies have defined both of PLD and PA as important players in the regulation of mTORC1 activity in response to serum, growth factors and amino acids (123,131). In addition, sustained activation of PKC reduced the interaction of

mTOR and its endogenous inhibitor FKBP38, suggesting that PA induced by activation of PKC might be involved in the process. Thus, our results raise the possibility that the accumulation of PA induced by activation of PKC trans-compartmentally activates the mTOR in the lysosomes.

In order to test this hypothesis, we plan to employ in vitro reconstitution assays. We will stimulate HEK293 cells with PMA and then isolate lysosomes by using the Lysosome Enrichment Kit according to the manufacturer's instructions and isolate the cPKC-containing endosomes using the centrifugation method (Optiprep) as previously described by our lab (9). Next we will study if putting the two fractions together is sufficient to stimulate phosphorylation of exogenously added p70S6K. Fractions from cells without PMA treatment will be used as control. To confirm that PA is not in the lysosomes but is required for the activation of mTORC1, we will add exogenously PA in to the lysosomes fraction with PMA treatment directly and then check if it is enough to phosphorylate exogenously added p70S6K. To confirm that the mTOR- containing lysosomes are required for the activation, we will test if adding exogenously PA into the cPKC-containing endosomes fraction is sufficient to phosphorylate exogenously added p70S6K.

6.4. Confirm the role of PKCα in regulation of mTORC1 and PI3K/Akt in animal models.

Since we identified the critical role of PKCα in the regulation of mTORC1 in NSCLC cell lines with mtEGFR, it is important to confirm what we have found in animal models. We plan to examine the activities of mTORC1 and PI3K/Akt in the tumors from HCC827 xenografts treated with vehicle or cPKC inhibitor, Enzastaurin. We will analyze the phosphorylation of p70S6K and Akt by western blotting and IHC in the tumors. Also the tumor size and volume respond to Enzautaurin compared with vehicle will be analyzed. Another animal model we could utilize is the transgenic mice that express an exon 19 deletion mutant or the L858R mutant under the control of doxycycline developed by Politi *et. al.* (151).

6.5. Study PKCα as a target for NSCLC already developed secondary mutation in mtEGFR.

NSCLC patients with mtEGFR show good response to tyrosine kinase inhibitor, such as gefitnib and erlotinib(139,140), but most of the patients with initial response to gefitinib or erlotinib ultimately develop resistant to the inhibitors (141). There are several identified mechanisms underlying the acquired resistance and almost 50% of the acquired resistance developed is related to the appearance of the secondary mutation (T790M) in tmtEGFR (142), which attenuates the binding efficacy of TKI to the mtEGFR (143). Thus, it is emergent to investigate the downstream signaling of mtEGFR and find potential target for personalized treatment of lung cancer.

Our results indicate that PKCα is the major mediator downstream of mtEGFR, regulating mTORC1 and PI3K/Akt signaling pathways, thus targeting PKCα could benefit the NSCLC patients who already developed secondary mutation (T790M) in mtEGFR.

6.5.1. Investigate the role of PKCα in regulating mTORC1 and PI3K signaling pathways in cell lines with the secondary mutation (T790M) in mtEGFR

HCC827-EPR and H1975 could be used here as the cell model, which has constitutively active EGFR mutation and the acquired T790M secondary mutation. cPKC inhibitor, Go6976 and Enzastaurin will be used to identify the role of cPKC in regulation of mTORC1 and PI3K signaling. In addition, siRNA and CRISPR approaches will be used to further confirm that.

6.5.2. Confirm the role of PKCα in regulation of mTORC1 and PI3K/Akt in transgenic mice with secondary mutation in EGFR (T790M)

If we could confirm that PKCα is the key mediator in regulation of mTORC1 and PI3K signaling in HCC827-EPR and H1975, then next we need to confirm that in mouse model expressing constitutively active EGFR mutant and EGFR T790M, which has been reported before (152). The transgenic mouse will be treated with vehicle or Enzastaurin. The tumor size and weight of those moues will be examined to evaluate the role of PKCα in tumor growth and progression associated with secondary EGFR mutation. In addition, tumors from the mouse will be analyzed to study if inhibition of cPKC reduces mTORC1 and PI3K/Akt signaling.

6.5. Conclusion

In conclusion this work provides new insight into the regulation of mTORC1 by PKC and identified PKCα as a major mediator in regulation of both mTORC1 and PI3K/Akt signaling pathways in response to mtEGFR. However, this study is only done in one or two cells. To further understand regulation of PKC and confirm the critical role of PKCα in cancer biology, we need to test our hypothesis in animal models.

Figures

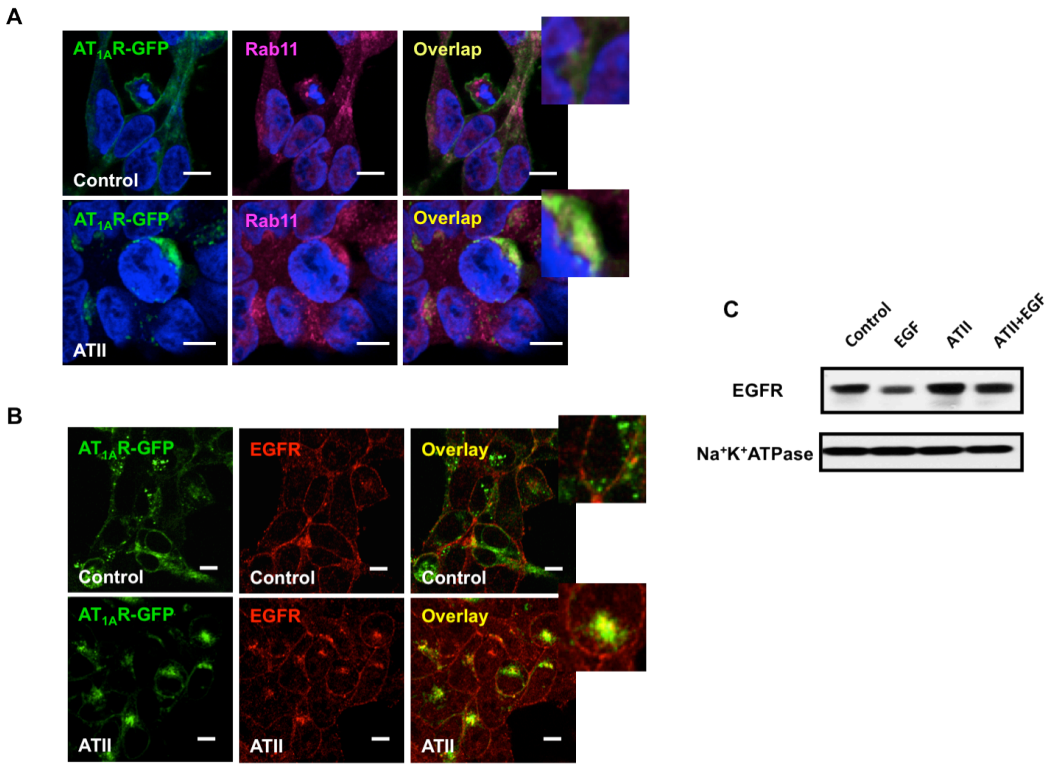


FIGURE 1. Effects of ATII on localization and fate of EGFR. A, HEK293 cells stably transfected with $AT_{1A}R$ (green) were serum starved for 5 hours followed by 100nM ATII or vehicle for 1 hour. After fixation and permeabilization, location of $AT_{1A}R$ (green) and endogenous Rab11 (red) were determined by immunofluorescence, and cells were analyzed by confocal microscopy (ZEISS 510). B, HEK293 cells stably transfected with $AT_{1A}R$ (green) were serum starved for 5 hours followed by 100nM ATII or vehicle for 1 hour. After fixation and permeabilization, endogenous EGFR (red) was determined by immunofluorescence, and cells were analyzed by confocal microscopy (ZEISS 510). C, HEK293 cells stably transfected with $AT_{1A}R$ were serum starved for 5 hours and treated with 2ng/ml of EGF for 3 hour with or without 1 hour pretreated with 100nM ATII. Protein level of EGFR was determined by Western Blotting. Blots were stripped and reprobed for Na^+K^+ATPase to normalize for loading. These results are representative of three independent experiments. Pictures are representative of at least three experiments.

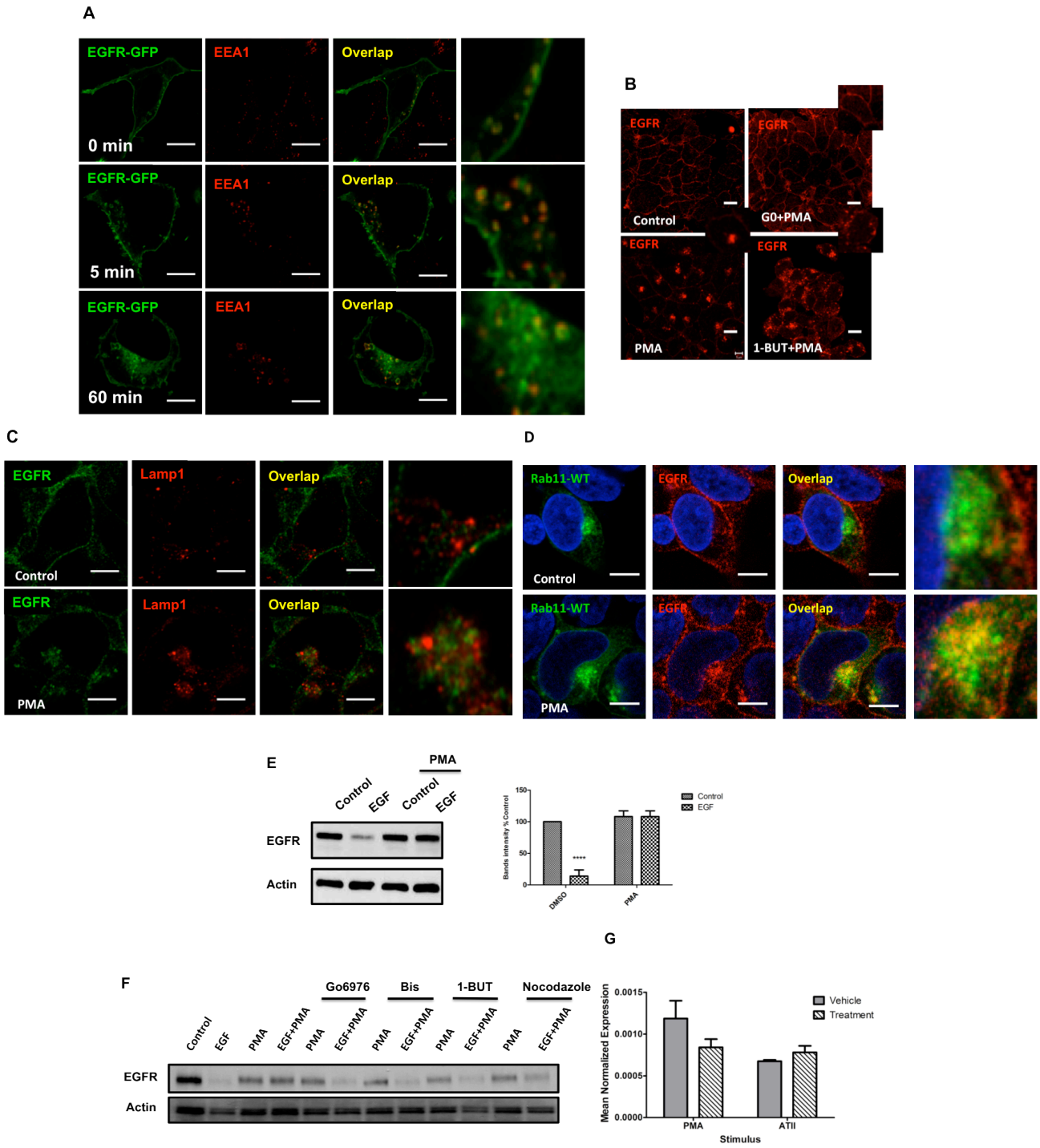

A
EGFR-GFP
EEA1
Overlap
0 min
5 min
60 min
B
EGFR
Control
G0+PMA
PMA
1-BUT+PMA
C
Lamp1
D
Rab11-WT
E
PMA
Control
EGF
Actin
Bands intensity %Control
DMSO
F
Go6976
Bis
1-BUT
Nocodazole
EGF+PMA
G
Vehicle
Treatment
Mean Normalized Expression
Stimulus
ATII

FIGURE 2. ATII-induced sequestration and protection of EGFR loss require the pericentrion. A, HEK293 cells were transfected with WT-EGFR-GFP. After 24 hours, cells were starved for 5 hours and then treated with 100nM PMA for 5 or 60 min. After fixation and permeabilization, location of EGFR (green) and endogenous EEA1 (red) were determined by immunofluorescence, and cells were analyzed by confocal microscopy (Leica TSC SP8). B, HEK293 cells were starved for 5 hours and then pretreated with Gö6976, 1-butanol or vehicle followed by 100nM PMA or vehicle for 1 hour. After fixation and permeabilization, endogenous EGFR (red) was determined by immunofluorescence, and cells were analyzed by confocal microscopy (ZEISS 510). C, HEK293 cells were starved for 5 hours and then treated with 100nM PMA or vehicle for 1 hour. After fixation and permeabilization, endogenous EGFR (green) and Lamp1 (red) were determined by immunofluorescence, and cells were analyzed by confocal microscopy (Leica TSC SP8). D, HEK293 cells were transfected with Rab11-GFP. After 24 hours, cells were starved for 5 hours and then treated with 100nM PMA or vehicle for 1 hour. After fixation and permeabilization, location of Rab11 (green) and endogenous EGFR (red) were determined by immunofluorescence, and cells were analyzed by confocal microscopy (Leica TSC SP8). E, HEK293 cells starved for 5 hours and then treated with 100nM PMA or vehicle for 1 hour following with 5ng/ml EGF or vehicle for 3 hours. Protein levels of EGFR and actin were determined by western blotting. F, HEK293 cells starved for 5 hours and then were pretreated with Gö6976, Bis, 1-butanol or vehicle followed with 100nM PMA for 1 hour and then treated with 5ng/ml EGF or vehicle for 3 hours. Protein levels of EGFR and actin were determined by western blotting. G, HEK293 cells or HEK293 cells stably overexpressing $AT_{1A}R$ were starved for 5 hours and then treated with vehicle, 100nM PMA or 100nM ATII for 1 hour. After treatments, cells were collected immediately and the mRNA level of EGFR were determined by real-time PCR. Pictures are representative of at least three experiments. E: **** $P<0.0001$ compared to control, two-way ANOVA.

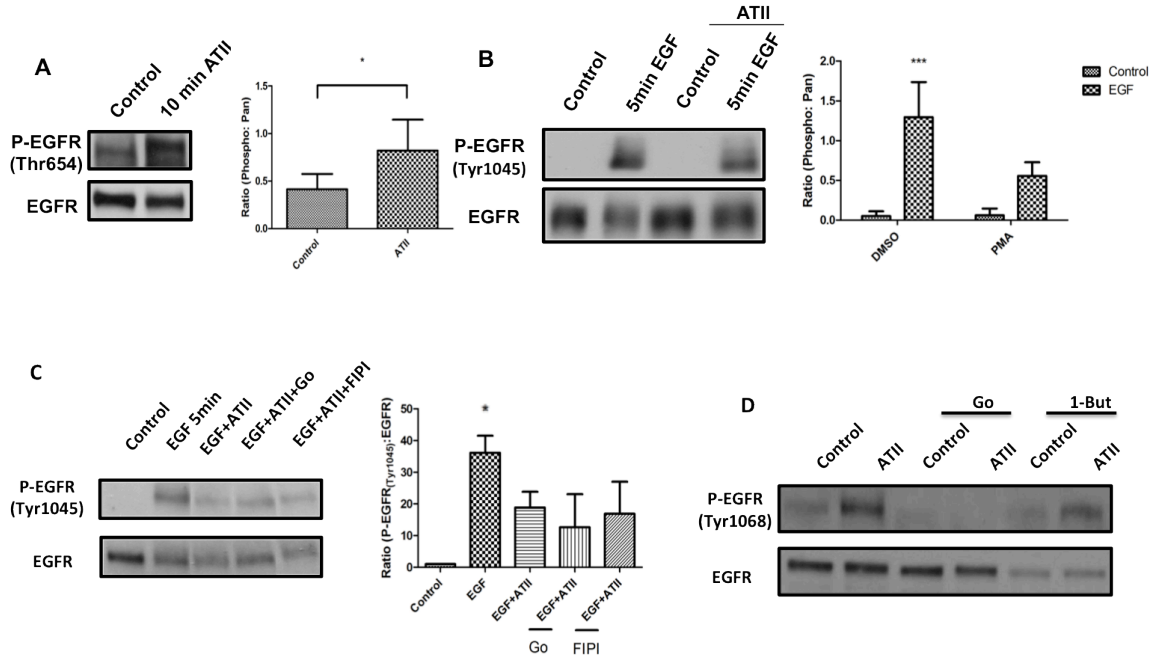


FIGURE 3. Effects of ATII on phosphorylation of EGFR. A, HEK293 cells stably transfected with $AT_{1A}R$ were serum starved for 5 hours followed by 100nM ATII for 10 min. Phosphorylation of EGFR on Thr-654 (P-Thr654) was determined by western blotting. Blots were stripped and reprobed for total EGFR to normalize for loading. B, HEK293 cells stably transfected with $AT_{1A}R$ were serum starved for 5 hours and treated with 5ng/ml of EGF for 5 min with or without 1 hour pretreated with 100nM ATII. Phosphorylation of EGFR on Tyrosine 1045 (P-Tyr1045) was determined by western blotting. The blots were stripped and reprobed for total EGFR to normalize for loading. C, HEK293 cells stably transfected with $AT_{1A}R$ were serum starved for 5hours and then pretreated with Gö6976, FIPI or vehicle followed with 100nM ATII or vehicle for 1 hour and then treated with 5ng/ml EGF for 5 min. P-Tyr1045 and EGFR were determined by western blotting. D, HEK293 cells stably transfected with $AT_{1A}R$ were serum starved for 5hours and then pretreated with Gö6976, 1-butanol or vehicle followed with 100nM ATII or vehicle for 5min, P-Tyr1068 and EGFR were determined by western blotting. For all figures, * $p < 0.05$, ***$p < 0.001$ from at least three independent experiments.

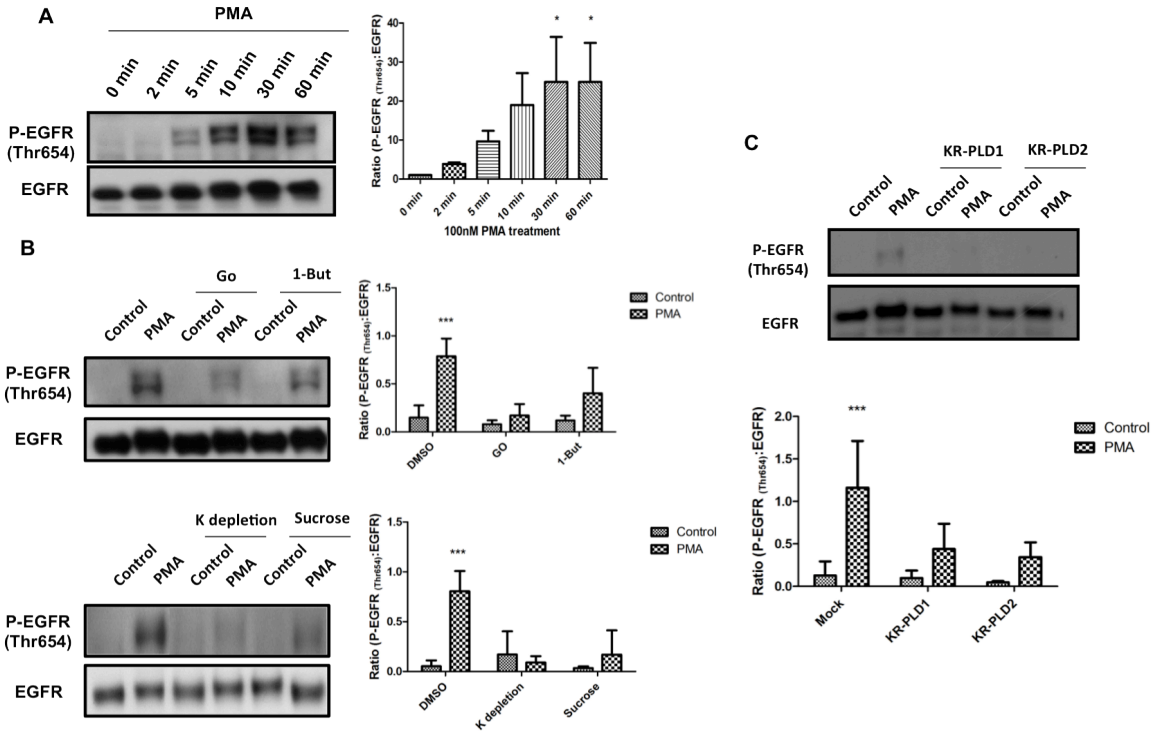

A
PMA
0 min
2 min
5 min
10 min
30 min
60 min
P-EGFR (Thr654)
EGFR
Ratio (P-EGFR (Thr654):EGFR)
100nM PMA treatment
B
Go
1-But
Control
PMA
DMSO
GO
1-But
K depletion
Sucrose
K depletion
Sucrose
C
KR-PLD1
KR-PLD2
Mock
KR-PLD1
KR-PLD2
D
PMA pretreatment
2 min
5 min
10 min
30 min
60 min
EGF
P-EGFR (Tyr1045)
F
2min EGF
2 min EGF
P-EGFR (Tyr1068)
Actin
E
Go
1-BUT
FIPI
5 min EGF

FIGURE 4. Effects of PMA on phosphorylation of EGFR and the role of the pericentrion. A, HEK293 cells were serum starved for 5 hours followed by 100nM PMA for 2 min, 5 min, 10 min, 30 min or 60 min. Phospho-Thr654 and total EGFR were determined as described above. B. HEK293 cells were starved for 5 hours and then pretreated with vehicle, Gö6976, 1-butanol, depleted of potassium (K-), or preincubated 400mM sucrose followed by 1-hour 100nM PMA treatment. The procedure for potassium-depletion is as described previously (30). Levels of P-Thr654 and total EGFR were determined as described. C, HEK293 cells were transfected with dominate negative constructs of PLD1 or PLD2. After 24 hours post-transfection, cells were starved for 5 hours and then treated with 100nM PMA for 1 hour. Levels of P-Thr654 and total EGFR were determined as shown before. D, HEK293 cells were starved for 5 hours and then pretreated with 100nM PMA for the indicated time followed by 5min 5ng/ml EGF treatment. Phosphorylation of EGFR on Tyrosine 1045 (P-Tyr1045) and total EGFR were determined as described. E, HEK293 cells were starved for 5 hours and then pretreated with Gö6976, 1-butanol, FIPI, or vehicle followed with 100nM PMA or vehicle for 1 hour and then treated with 5ng/ml EGF or vehicle for 5 min. P-Tyr1045 and EGFR were determined by western blotting. F, HEK293 cells were starved for 5 hours and then pretreated with vehicle or 100nM for 1 hour followed by treatment with 10ng/ml EGF for 2 min. Phospho-Tyr1068 and EGFR were determined by western blotting. For all figures, * $p < 0.05$, ***$p < 0.001$ from at least three independent experiments.

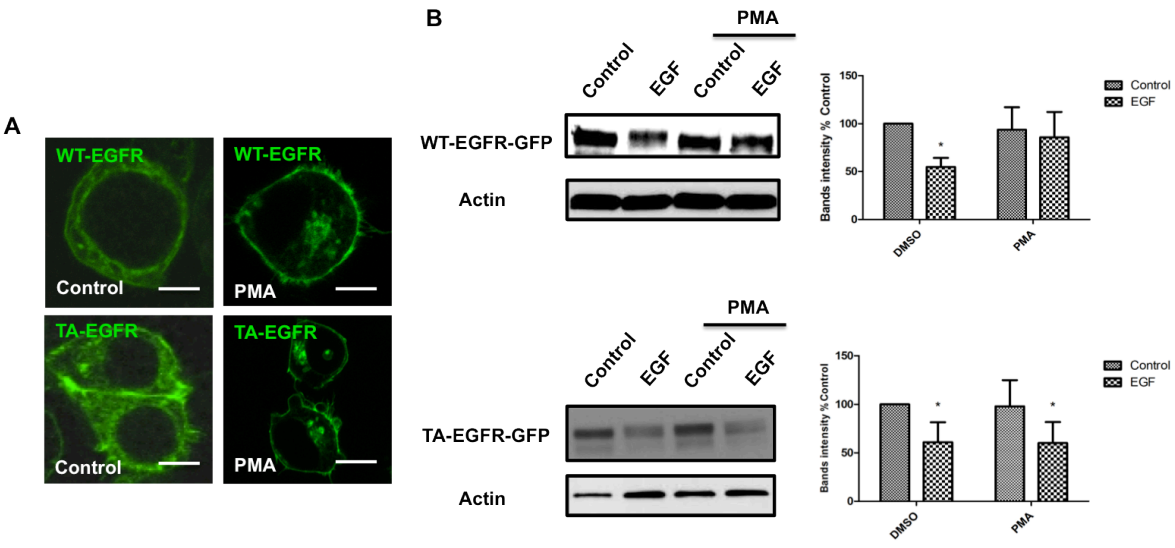


FIGURE 5. Effects of PMA on EGFR T654A mutant. A, HEK293 cells were transfected with WT-EGFR-GFP or TA-EGFR-GFP. 24 hours after transfection, cells were starved for 5 hours and then treated with 100nM PMA or vehicle for 1 hour. After fixation, cells were analyzed by confocal microscopy (ZEISS 510). B, HEK293 cells were transfected with WT-EGFR-GFP or TA-EGFR-GFP. 24 hours after transfection, cells were starved for 5 hours and then pretreated with 100nM PMA or vehicle for 1 hour followed by 5ng/ml EGF for 3 hours. The levels of EGFR, EGFR-GFP and actin were determined by western blotting. For all figures, * $p < 0.05$, from at least three independent experiments.

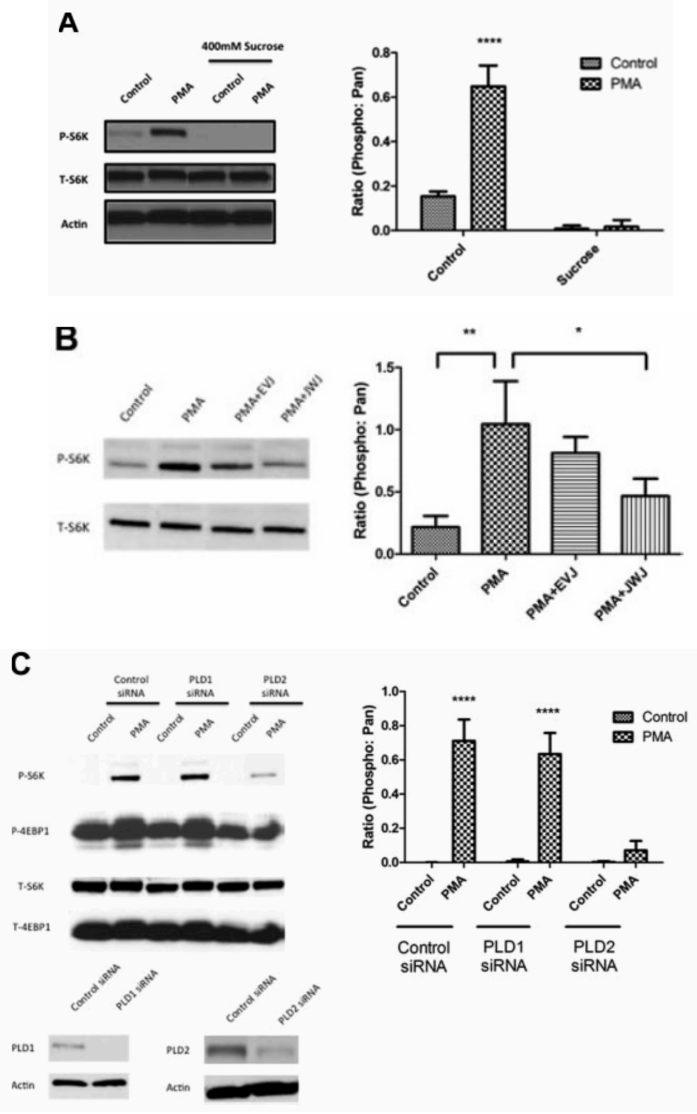


FIGURE 6. S6K activation by PMA requires PKC internalization. HEK cells were plated and 24h later were serum starved overnight. Following starvation, cells were pretreated with (**A**) sucrose or (**B**) the PLD inhibitors EVJ (PLD1) and JWJ (PLD2) prior to stimulation with vehicle or PMA as shown. (**C**) HEK cells were treated with negative control (AS), PLD1 or PLD2 siRNA for 48h prior to stimulation with PMA (100nM) or 20% serum for 1h. In all cases, total lysates were prepared and immunoblotted. Pictures are representative of at least three experiments (A, C: **** $P<0.0001$ compared to control, two-way ANOVA; B - * $P<0.05$, ** $P<0.01$ one-way ANOVA)

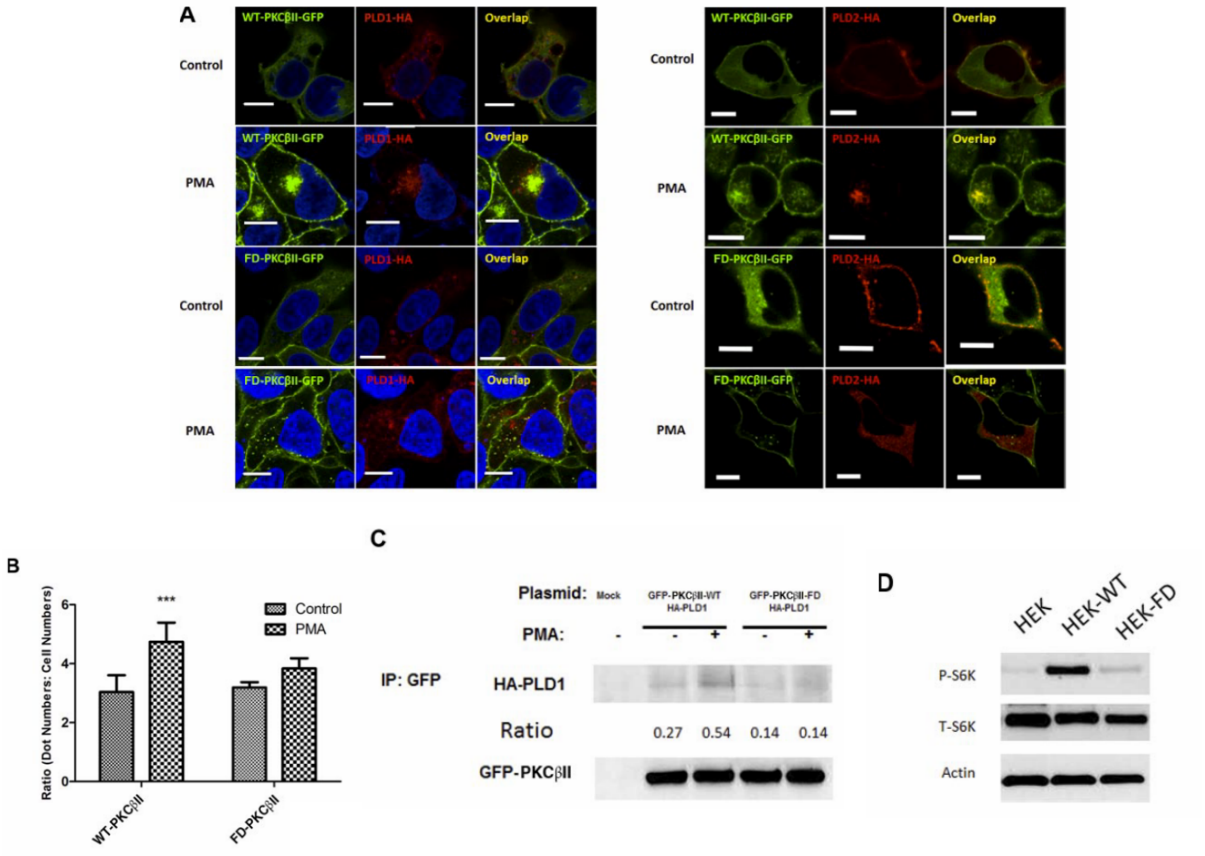


FIGURE 7. Wild-type PKCβII but not the F666D mutant interacts with PLD and enhances mTORC1 activity in HEK cells. (**A**) HEK and A549 cells were co-transfected with wild-type PKCβII (WT) or PKCβII-F666D mutant (FD) and either PLD1 or PLD2. Cells were serum-starved and stimulated with vehicle or PMA for 1h as shown. PKC and PLD localization was determined by confocal microscopy. (**B**) Cells were co-transfected with wild-type PKCβII (WT) or a PKCβII-F666D mutant (FD) and PLD1, serum-starved and stimulated with vehicle or PMA for 1h. The relative proximity of PKC and PLD1 was assessed by PLA as described in 'Experimental Procedures'. Quantification is of multiple fields from at least two independent experiments (**P<0.01 compared to control, two-way ANOVA) (**C**) Cells were co-transfected with GFP-tagged wild-type PKCβII (WT) or a PKCβII-F666D mutant (FD) and PLD1, serum-starved and stimulated with vehicle or PMA for 1h. GFP was used for immunoprecipitation and the levels of PLD1 and PKC-GFP were assessed in immunoprecipitates. (**D**) HEK cells, HEK cells stably expressing PKCβII-WT and PKCβII-F666D were plated and after 24h later, total lysates were prepared and immunoblotted as shown. The blots shown are representative of at least three independent experiments.

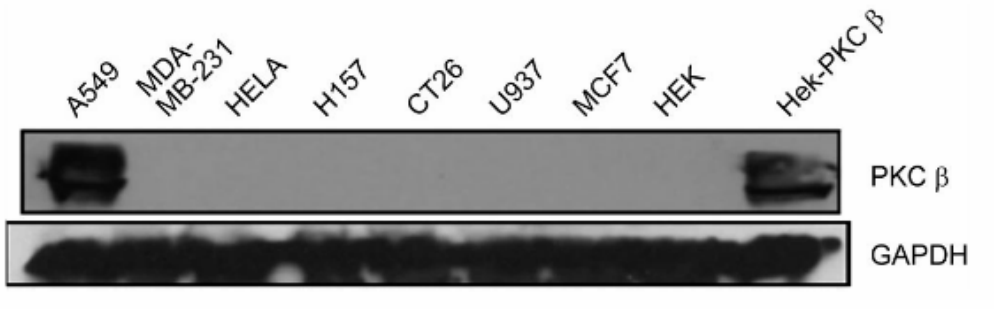


FIGURE 8. A549 cells possess high endogenous PKCβ levels. Total cell lysates were prepared from the cell lines and immunoblotted for PKCβ. HEK cells stably expressing wild-type PKCβII were used as a positive control. Blots shown are representative of three independent experiments.

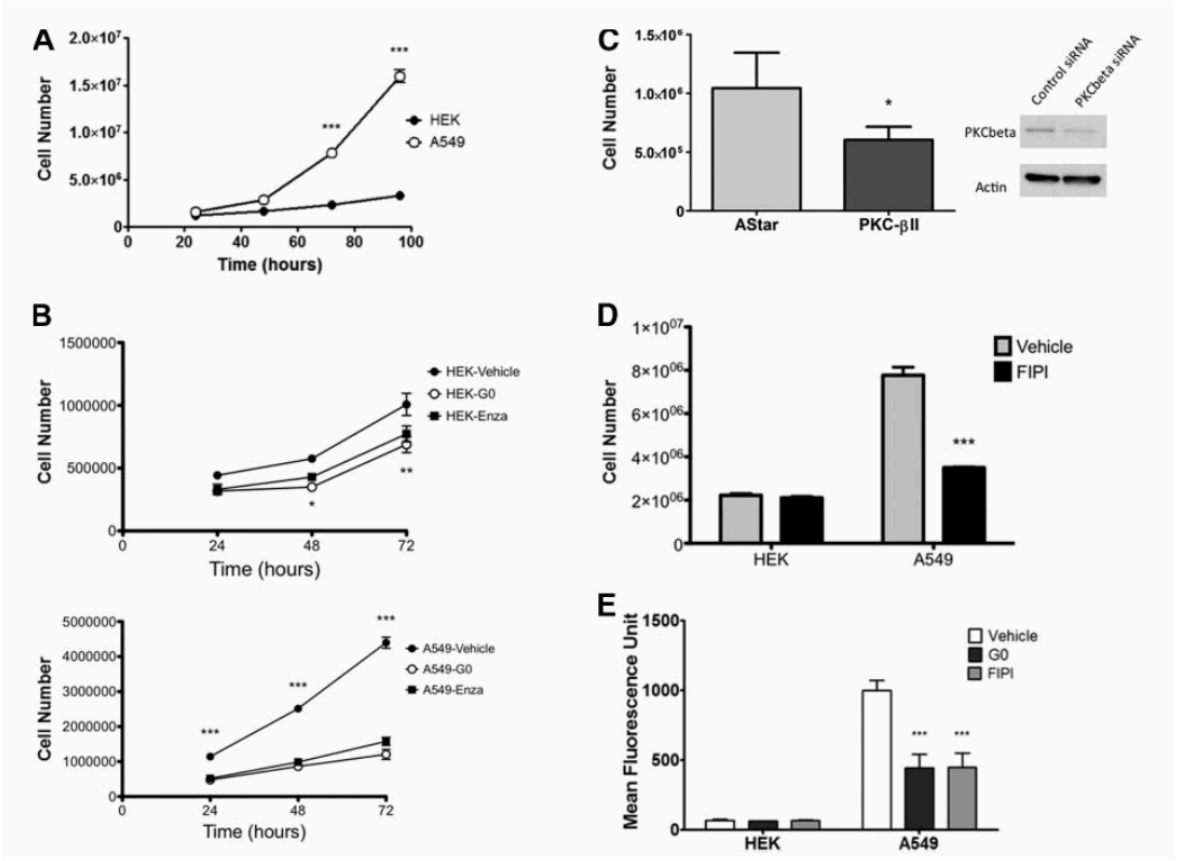


FIGURE 9. Increased growth and migration of A549 cells is PKC and PLD-dependent. (**A**) A549 and HEK cells were plated and cultured for the times indicated. At each time point, cells were trypsinized and counted in triplicate (*** P<0.001 compared to HEK; two-way ANOVA); (**B**) HEK and A549 cells were cultured as shown in the presence of vehicle (DMSO), Go6976 (3μM) or enzastaurin (3μM). At the indicated times, cells were trypsinized and counted in triplicate (* P<0.05, ** P<0.01, *** P<0.001 compared to vehicle; two-way ANOVA); (**C**) A549 cells were plated and transfected with negative control or PKCβ siRNA. 24h later, cells were placed in fresh media for a further 48h before cells were trypsinized and counted in triplicate. In each experiment, total lysates were prepared from matched samples and PKCβII levels were determined by immunoblot. (* P<0.05 compared to AStar, t-test n = 3) (**D**) HEK and A549 cells were cultured as shown in the presence of vehicle (DMSO) or FIPI (3μM) for 72h. Cells were trypsinized and counted in triplicate (*** P<0.001 compared to vehicle; two-way ANOVA) (**E**) HEK and A549 cell migration was analyzed by transwell assay. Cells were placed in the upper chamber and media with 10% FBS was placed in the lower chamber. Cells were allowed to migrate for 24h in the presence of vehicle (DMSO), Go6976 (3μM) or FIPI (3μM). Migrated cells were stained with fluorescent dye. (*** P<0.001 compared to vehicle; two-way ANOVA)

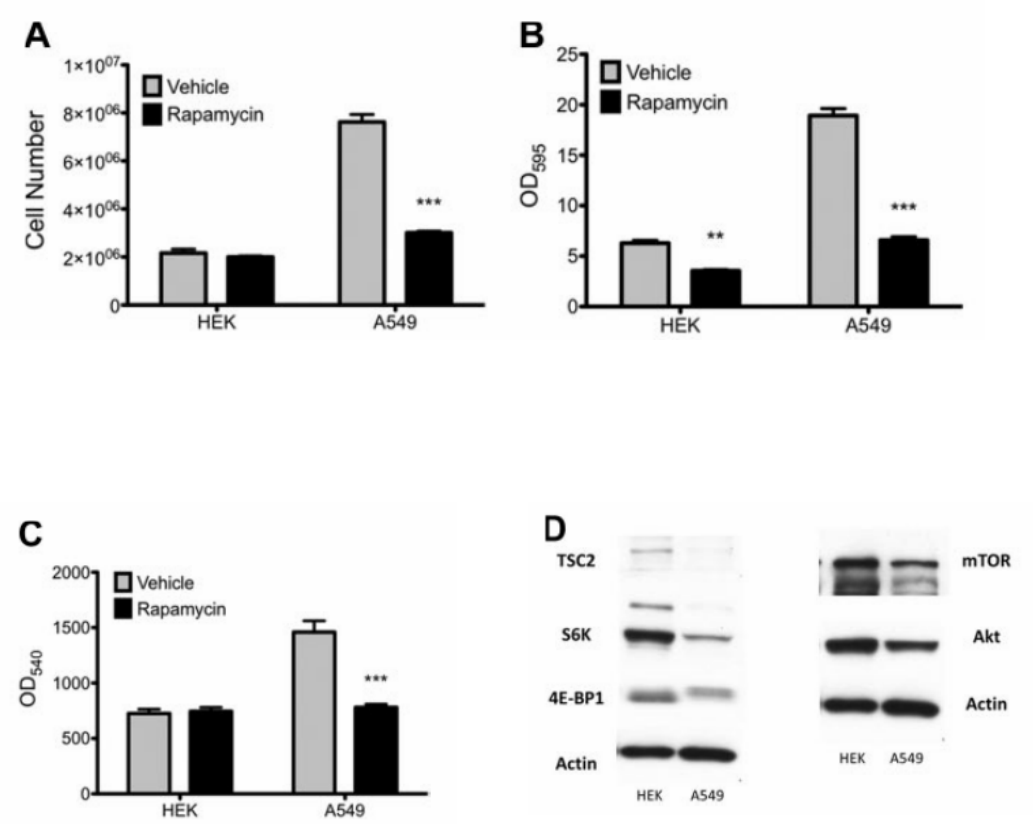


FIGURE 10. Increased growth and migration of A549 cells is mTOR-dependent. (**A**) HEK and A549 cells were cultured as shown in the presence of vehicle (DMSO) or rapamycin (100nM) for 72h. Cells were trypsinized and counted in triplicate; **(B**) Cells were treated as in (A) and cell growth was analyzed by MTT assay; (**C**) HEK and A549 cell migration was analyzed by transwell assay as described in 'Materials and Methods'. Cells were allowed to migrate for 24h in the presence of vehicle (DMSO) or rapamycin (100nM). (**D**) HEK and A549 cells were plated and allowed to grow for 24h. Total lysates were prepared and immunoblotted as shown with actin as loading control. Blots are representative of three independent experiments (** $P<0.01$, *** $P<0.001$ compared to vehicle; two-way ANOVA)

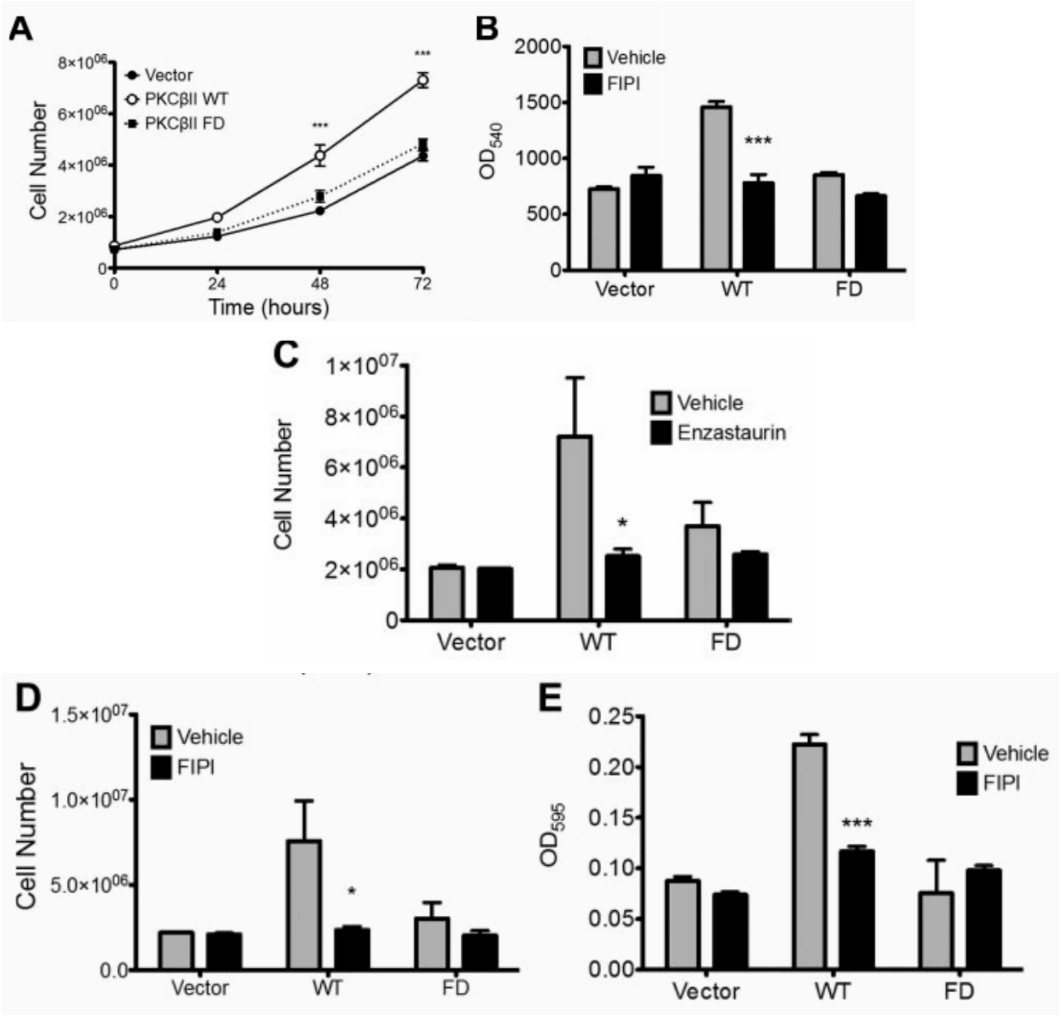


FIGURE 11. Stable overexpression of PKCβII **increases growth and migration of HEK in a PLD-dependent manner.** (**A**) HEK cells stably expressing vector, PKCβII-WT and PKCβII-F666D were cultured for the times shown. At the time points indicated, cells were trypsinized and viable cell numbers were counted (*** P<0.001 compared to HEK; two-way ANOVA); (**B**) Migration of HEK cells stably expressing vector, PKCβII-WT and PKCβII-F666D towards serum was assessed in the presence and absence of FIPI as described in 'Materials and Methods'. Cells were allowed to migrate for 24h in the presence of vehicle (DMSO) or FIPI. (**C**) HEK cells stably expressing vector, PKCβII-WT and PKCβII-F666D were cultured for 72h in the presence or absence of enzastaurin. After this time, cells were trypsinized and viable cell numbers were counted; (**D**) HEK cells stably expressing vector, PKCβII-WT and PKCβII-F666D were cultured for 72h in the presence or absence of FIPI. After this time, cells were trypsinized and viable cell numbers were counted; (**E**) HEK cells stably expressing vector, PKCβII-WT and PKCβII-F666D were cultured for 72h in the presence or absence of FIPI. After this time, viable cell number was assessed by MTT assay; (**E**) For all figures, * p <0.05, *** p<0.001 from at least three independent experiments)

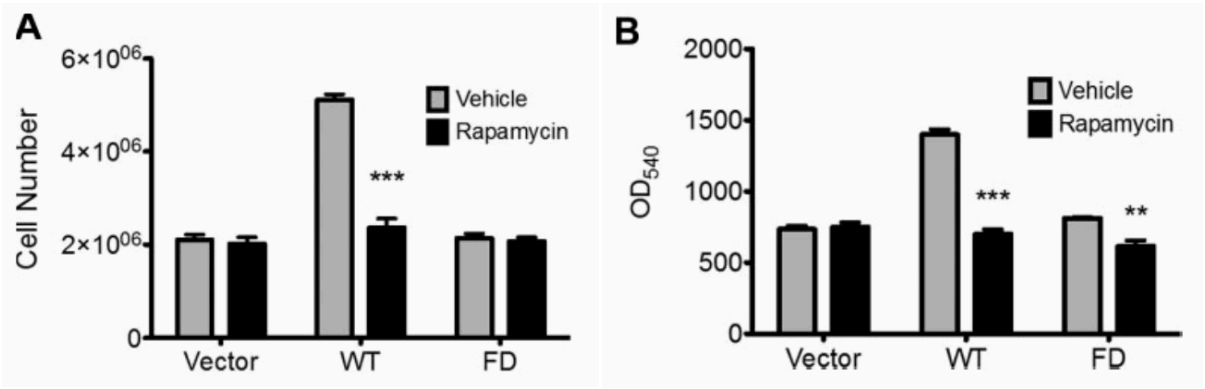
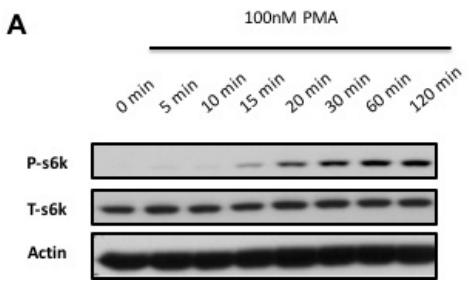
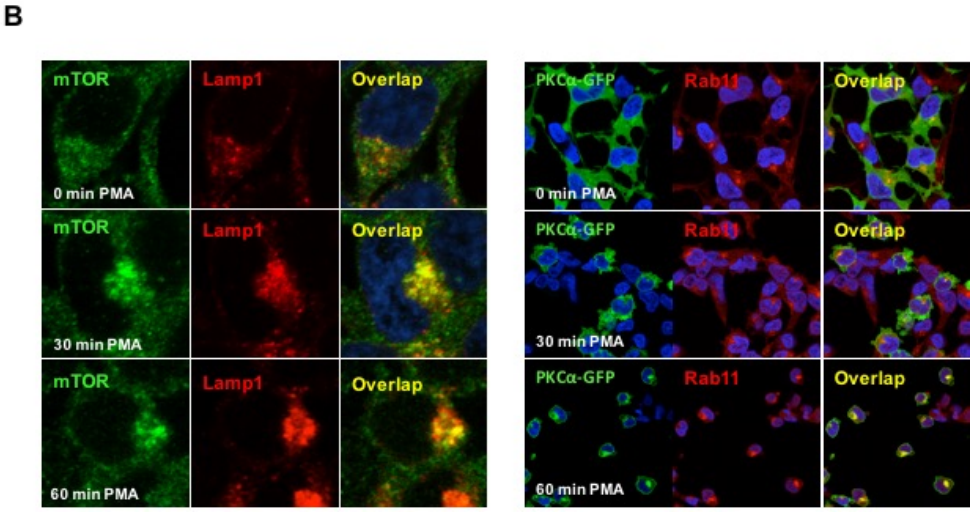
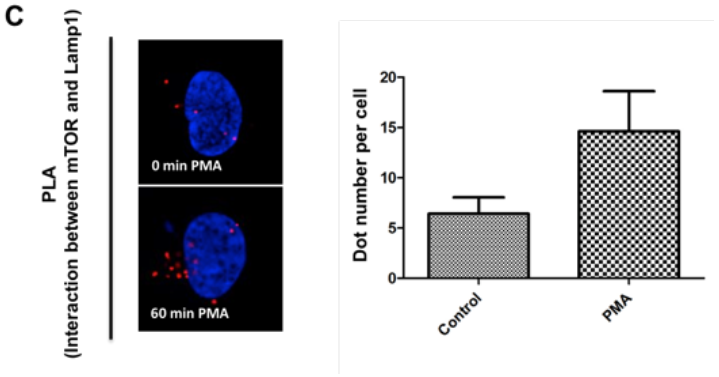


FIGURE 12. Enhanced growth and migration of HEK-PKCβII cells are dependent on mTOR activity. (**A**) HEK cells stably expressing vector, PKCβII-WT and PKCβII-F666D were cultured for 72h in the presence or absence of rapamycin and cell numbers were counted; (**B**) Cell migration was analyzed by transwell assay as described in 'Materials and Methods'. Cells were allowed to migrate for 24h in the presence of vehicle (DMSO) or rapamycin. (** P <0.02, *** P<0.001 compared to vehicle, two-way ANOVA)


A
100nM PMA
0 min
5 min
10 min
15 min
20 min
30 min
60 min
120 min
P-s6k
T-s6k
Actin


B
mTOR
Lamp1
Overlap
0 min PMA
30 min PMA
60 min PMA
PKCα-GFP
Rab11
Overlap
0 min PMA
30 min PMA
60 min PMA


C
PLA
(Interaction between mTOR and Lamp1)
0 min PMA
60 min PMA
Dot number per cell
20
15
10
5
0
Control
PMA

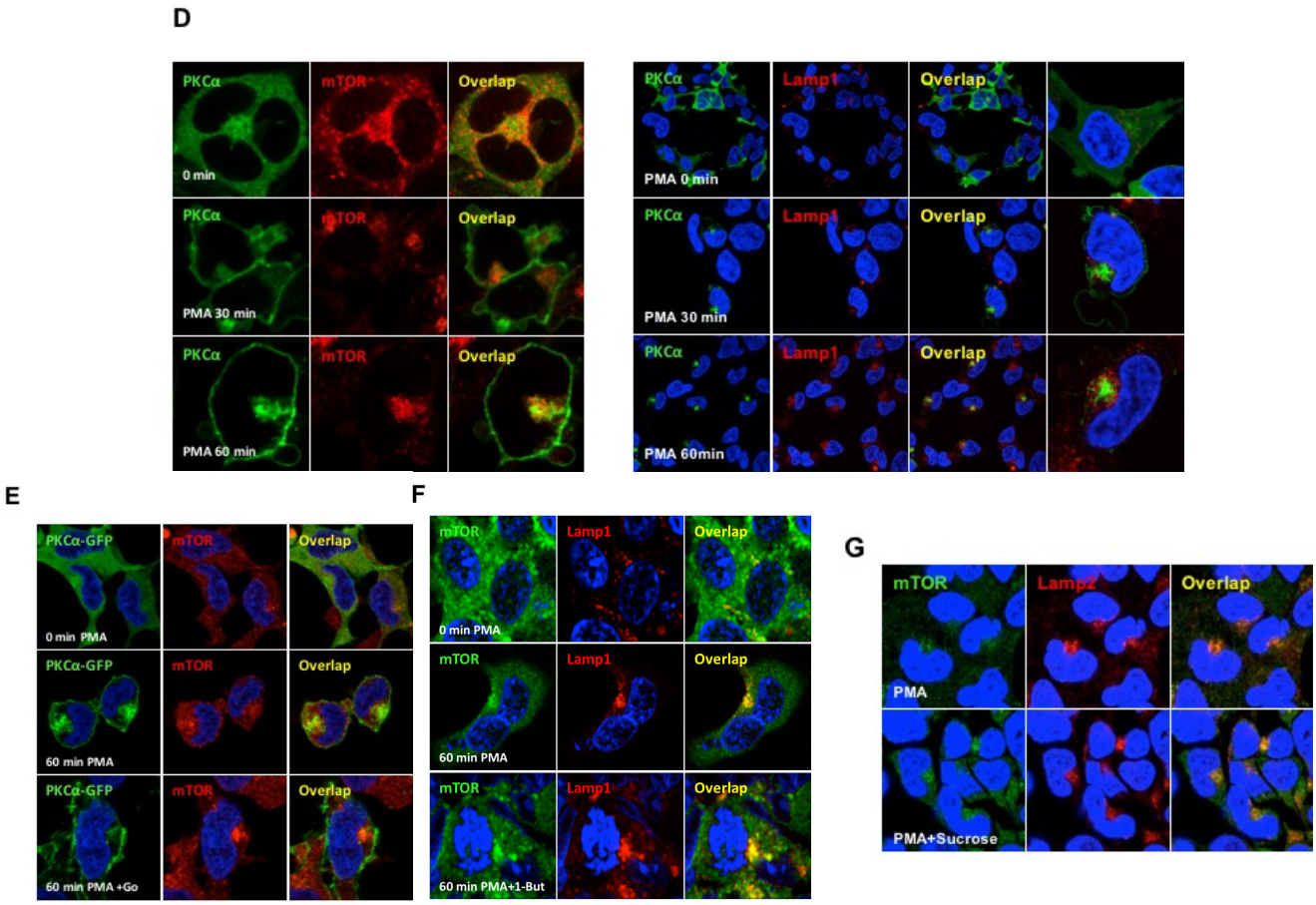


FIGURE 13. PMA induces the accumulation of mTOR in the perinuclear lysosomes, which is close to the cPKC-containing endosomes but two distinguished compartments. (**A**) HEK cells were starved for 5 hours and then treated with 100nM at indicated time; (**B**) HEK cells were serum starved following with PMA for 30 min or 60 min. After treatment cells were fixed and permeabilized, and then incubated with indicated primary antibodies. To check PKCα localization, HEK cells were first transfected with PKCα-GFP plasmid; (**C**) Hela cells were starved for 5 hours and then treated with 100nM PMA for 60 min. The relative proximity of mTOR and Lamp1 was assessed by PLA as described in methods; (**D**) HEK cells were transfected with PKCα-GFP and after transfection cells were starved for 5hours and treated with PMA, the staining of mTOR and Lamp1 was following the immunofluorescence protocol; (**E**) HEK cells were transfected with PKCα-GFP. After starvation, cells were pretreated with 3uM Go6976 for 1 hour following with 100nM PMA for 1 hour. (**F**) HEK cells were starved for 5hour, and then cells were pretreated with 3uM Bis for 1 hour following with 100nM PMA for 1 hour; (**G**) HEK cell were starved for 5 hours, and then cells were treated with hypertonic (400 mM) sucrose for 30min following with 100nM for 60 min.

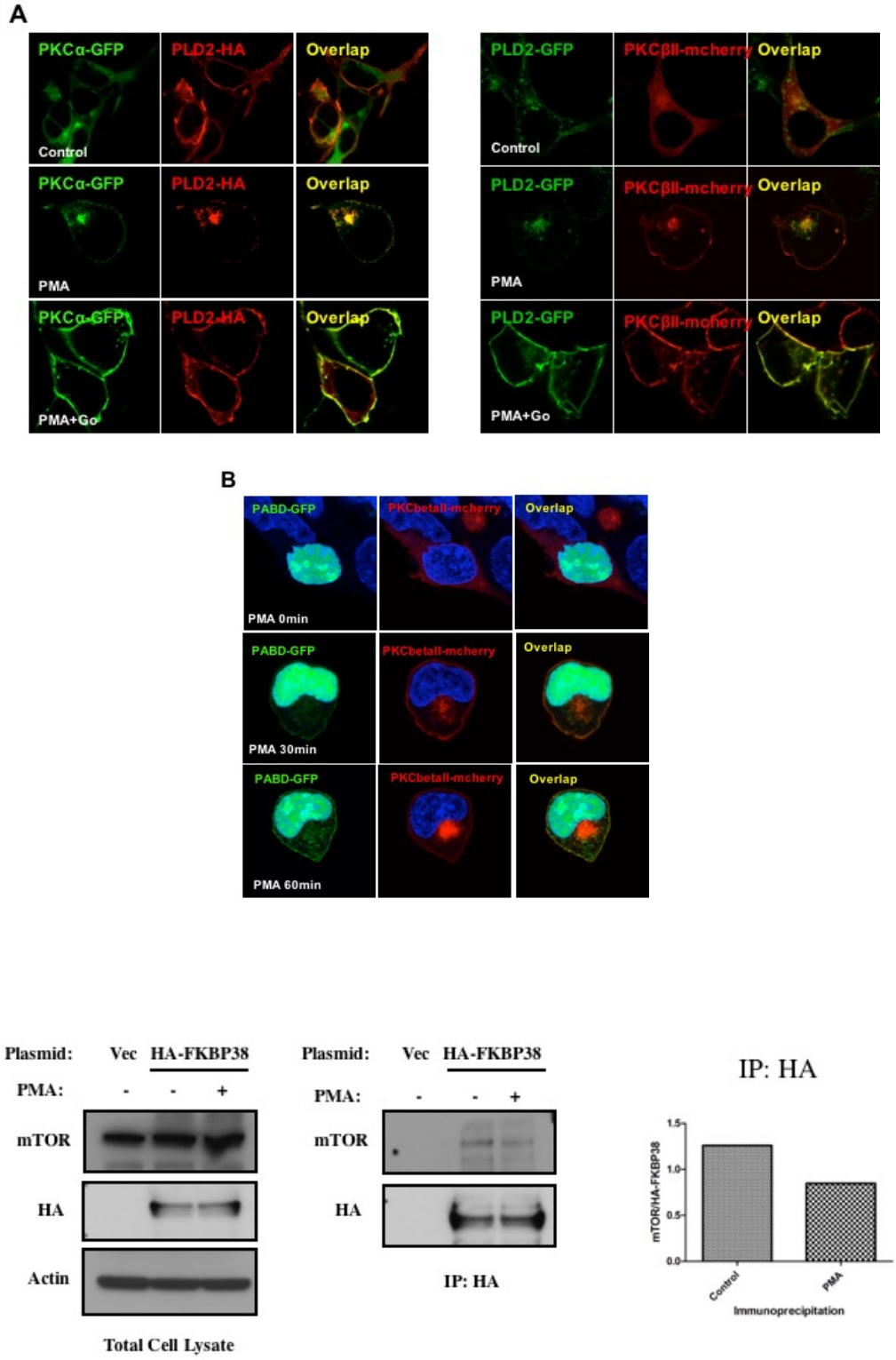
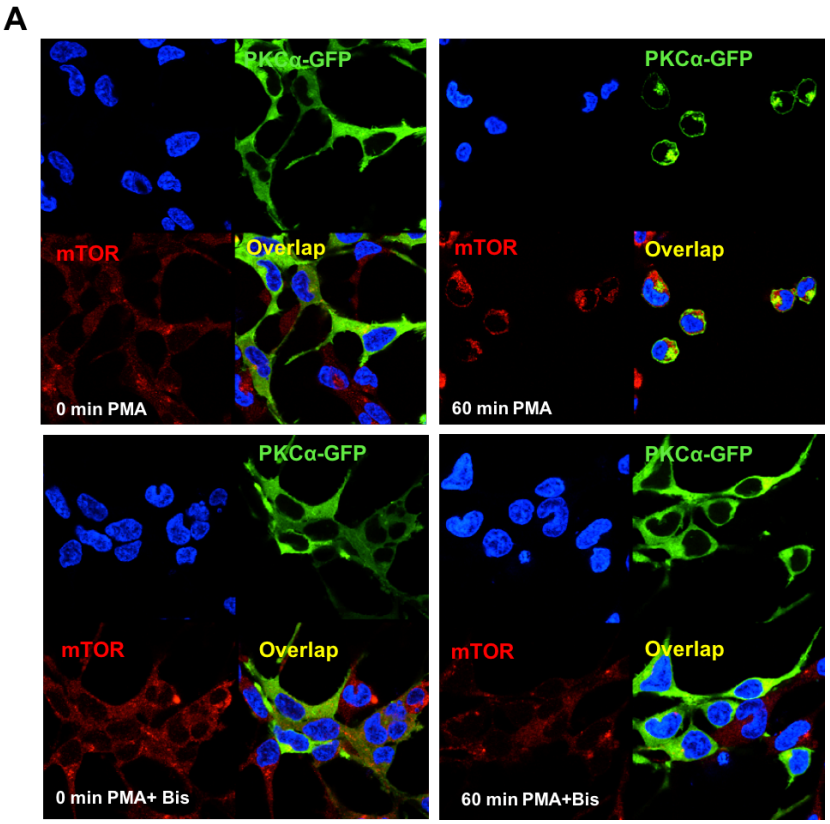
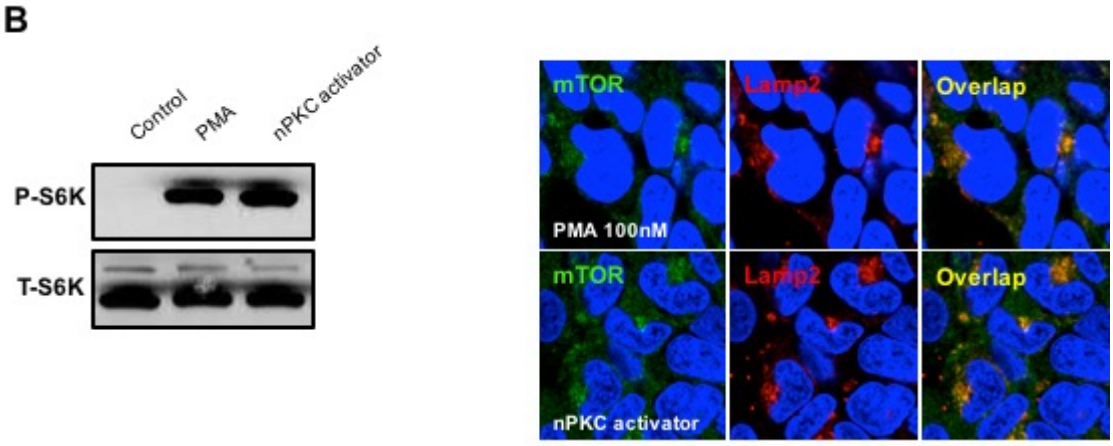


FIGURE 14. Upon sustained stimulation of PKC, PLD2 and PA accumulates in the cPKC-containing endosomes activates mTORC1 through displacing the endogenous inhibitor FKBP38 from mTOR. (**A**) HEK cells were co- transfected with PKCα-GFP and PLD2-HA or

PLD2-GFP and PKCβII-mcherry following with serum starvation and then 100nM PMA or Go6976 plus 100nM PMA. (**B**) HEK cells were transfected with PABD-GFP. After serum starvation, cells were treated with 100nM for 30 min or 60 min; (**C**) HEK cells were transfected with FKBP38-HA. After starvation, cells were treated with 100nM PMA and then the interaction of mTOR and FKBP38 was examined by immunoprecipitation with HA antibody.


A
PKCα-GFP
mTOR
Overlap
0 min PMA
PKCα-GFP
mTOR
Overlap
60 min PMA
PKCα-GFP
mTOR
Overlap
0 min PMA+ Bis
PKCα-GFP
mTOR
Overlap
60 min PMA+Bis


B
Control
PMA
nPKC activator
P-S6K
T-S6K
mTOR
Lamp2
Overlap
PMA 100nM
mTOR
Lamp2
Overlap
nPKC activator

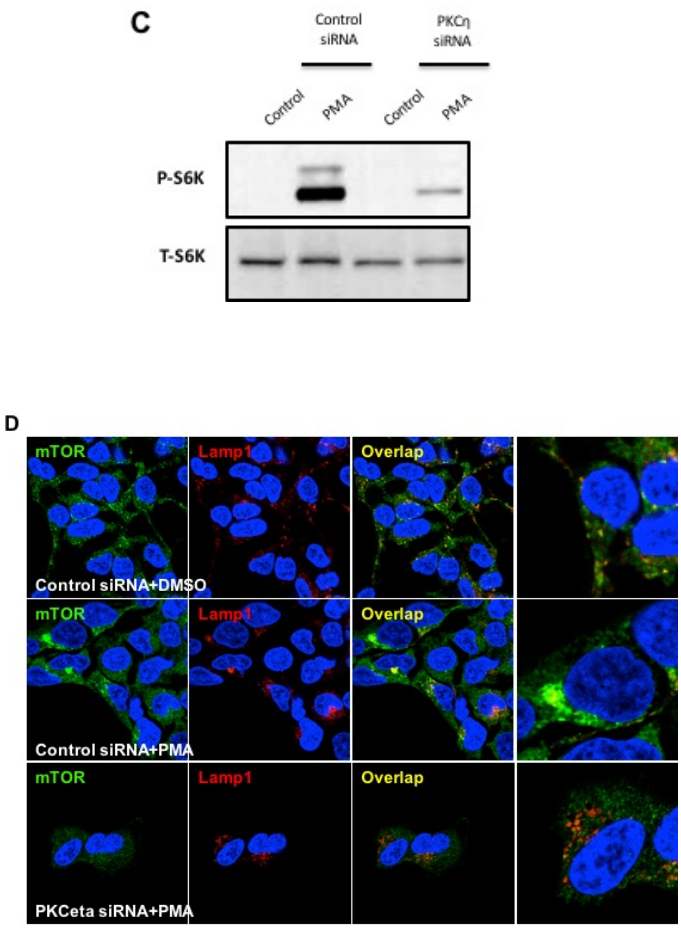


FIGURE 15 Novel PKC, PKCη, is required for mTORC1 activation by regulating mTOR translocalization. (**A**) HEK cells were serum starved for 5 hours and then treated with DMSO or 3uM Bis following with treatment of 100nM PMA or DMSO; (**B**) HEK cells were starved and then treated with 100nM PMA or the nPKC activator; (**C**) HEK cells were transfected with control siRNA or PKCη siRNA for 48 to 72 hours and treated with 100nM PMA for 60 min.

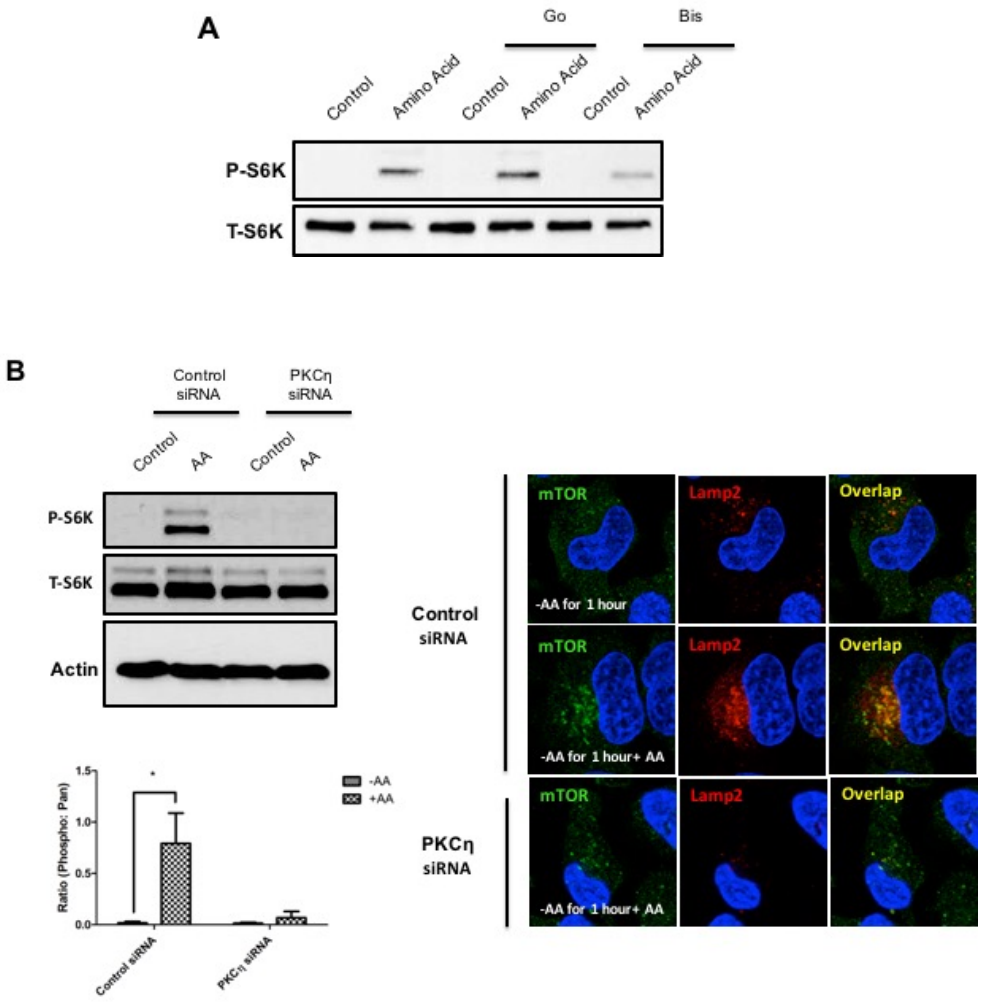


FIGURE 16. Amino acid utilizes PKCη to induce the activation and translocation of mTORC1. (**A**) Hela cells were amino acid starved and then pretreated with DMSO, Go6976 or Bis for 1 hour following with recovery of amino acid as described in the methods; (**B**) Hela cells were transfected with control siRNA or PKCη siRNA. After amino acid starvation, cells were recovered by adding extra amino acid in DMEM as described in the methods.

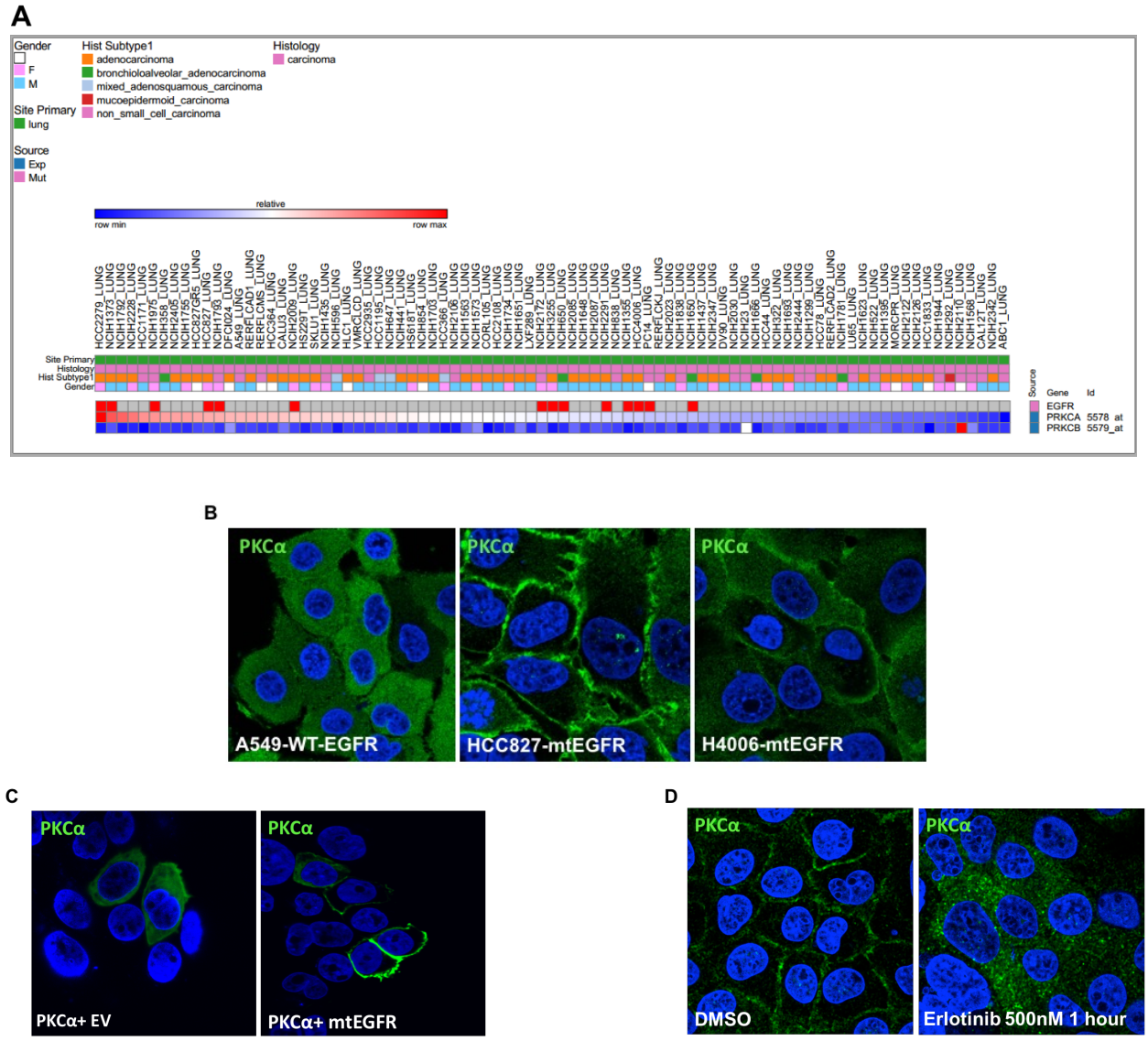


FIGURE 17. PKCα status in NSCLC (**A**) Affymetric U133-2 array was used here to examine the expression of mtEGFR, PKCα and PKCβ in lung cancer (**B**) A549, HCC827 and H4006 cells were serum starved for 5 hours and then immunofluorescence was performed to visualize endogenous PKCα; (**C**) H292 cells were co- transfected with PKCα-GFP and empty vector or PKCα –GFP and mtEGFR. After 24 hours, cells were serum starved for 5 hours; (**D**) HCC827 cells were starved 5 hours and treated with DMSO or 500nM erlotinib for 1 hour.

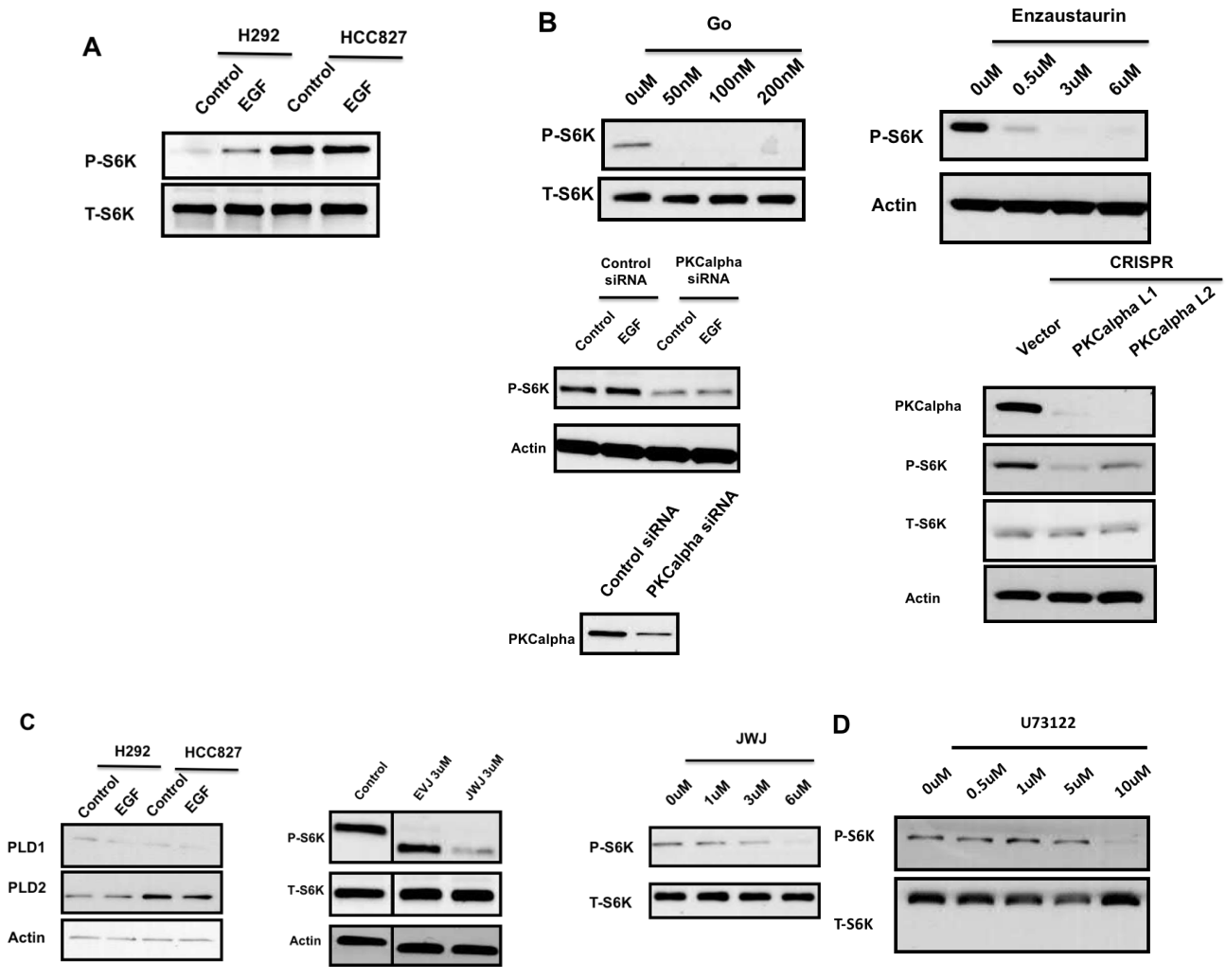


FIGURE 18. Activation of mTORC1 in NSCLC cell lines with mtEGFR requires PKCα and PLD2. (**A**) H292 cells and HCC827 cells were serum starved for 5 hours and treated with 10ng/ml EGF for 5 min as indicated; (**B**) For Go and Enzastaurin treatment, HCC827 cells were serum starved for 5 hours ant the treated with inhibitors at different concentration as indicated; For knockdown using siRNA, HCC827 cells were transfected with control siRNA or PKCα siRNA for 72 hours, and then serum starved for 5 hours following with 10ng/ml EGF for 5 min; for PKCα CRISPR cell lines, cells were starved for 5 hours and then protein levels were checked by western blotting; (**C**) H292 and HCC827 cells were serum starved for 5 hours following with the treatments as indicated; (**D**) HCC827 cells were serum starved for 5 hours and then treated with the inhibitor for 1 hour at different concentration as indicated.

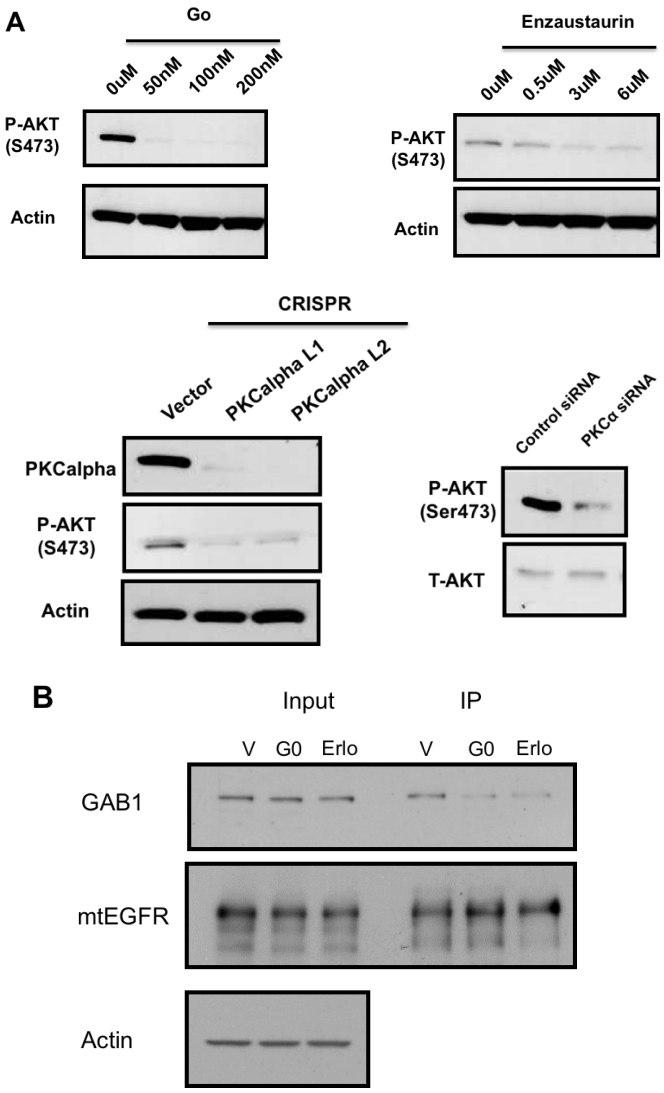


FIGURE. 19 (A) PKCα is involved in the activation of PI3K/Akt induced by mtEGFR in HCC827 (**A**) HCC827 cells were serum starved for 5 hours and then treated with inhibitors at different concentration as indicated. For PKCα CRISPR cell lines, cells were starved for 5 hours and then protein levels were checked by western blotting; (**B**) HCC827 cells were serum starved for 5 hours and then were treated with DMSO, Go 6976 or erlotinib, following with immunoprecipitation by using mtEGFR antibody.

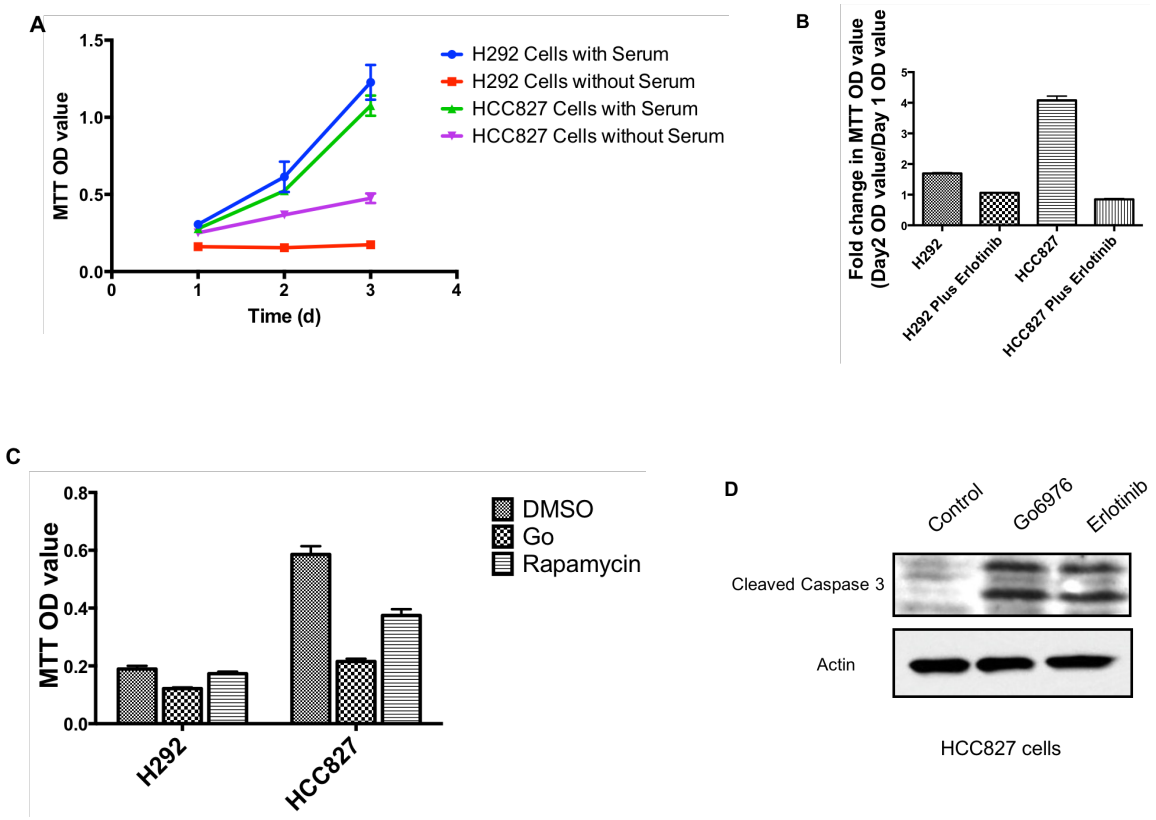


FIGURE 20. PKCα regulates cell survival and apoptosis in HCC827. (**A**) H292 and HCC827 cells were grown in medium with serum or without serum as indicated. MTT OD value was read at 1 day, 2 days, 3 days and 4 days after the feeding; (**B**) H292 cells and HCC827 cells were grown in medium without serum plus DMSO or 100nM erlotnib for 48 hours; (**C**) H292 and HCC827 cells were grown in medium without serum plus DMSO, 100nM Go, or 100nM erlotinib for 48 hours; (**D**) HCC827 cells were grown in serum free medium plus DMSO, 100nM Go6976, or 100nM erlotinib for 48 hours. The cleaved caspase 3 was detected by western blotting.

Supplemental Figures

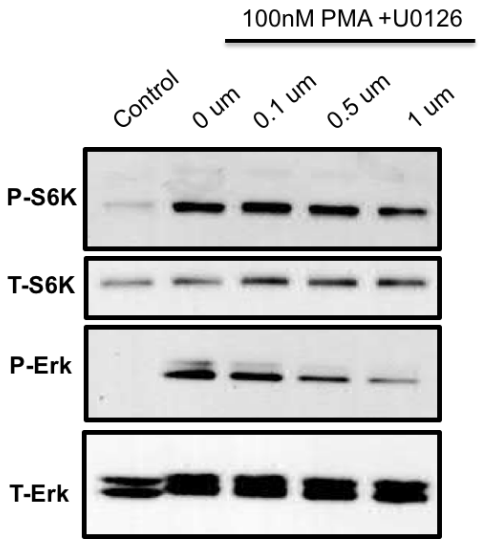


FIGURE S1. Activation of mTORC1 induced by PMA does not require MEK activity. HEK cells were serum starved for 5 hours and then treated with MEK inhibitor, U0126 for 1 hour at indicated concentration, following with 100nM PMA for 1 hour. Phosphorylation of S6K and Erk, and total protein levels of S6K and Erk were examined by western blotting.

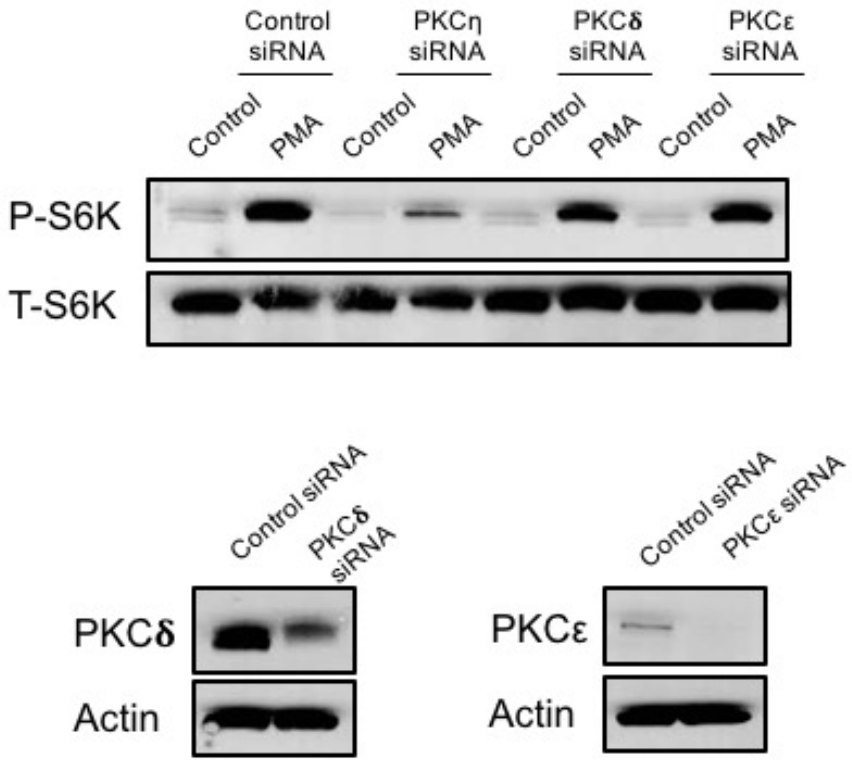


FIGURE S2. Activation of mTORC1 induced by PMA does not require PKCδ and PKCε. HEK cells were transfected with control siRNA, PKCη siRNA, PKCδ siRNA, or PKCε siRNA. After 72 hours, cells were serum starved for 5 hours and then treated with 100nM PMA for 1 hour. The protein levels were examined by western blotting.

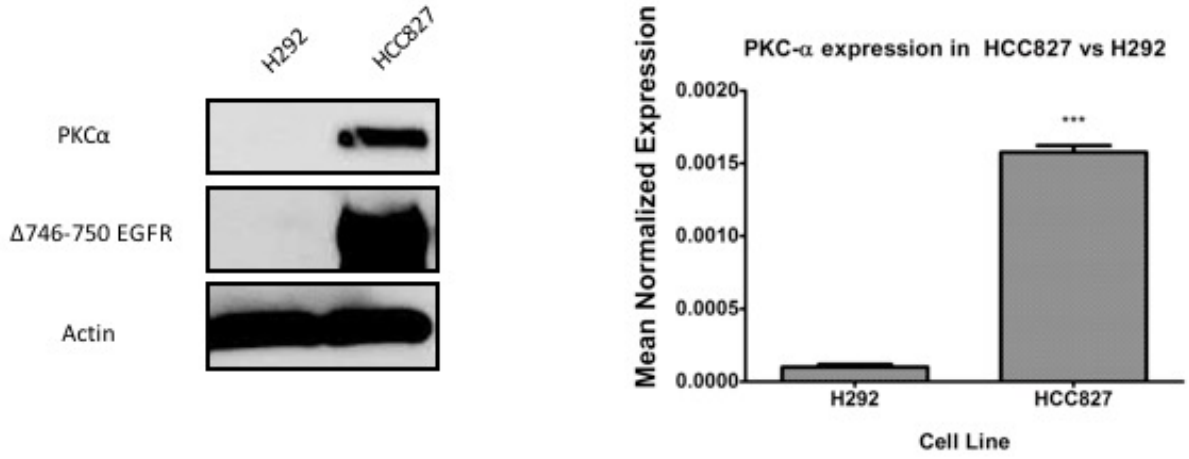


FIGURE S3. PKCα level in NSCLC cell lines. The protein level of PKCα was analyzed by western blotting in H292 and HCC827 cells. The mRNA levels of PKCα in H292 and HCC827 cells were examined by real-time PCR.

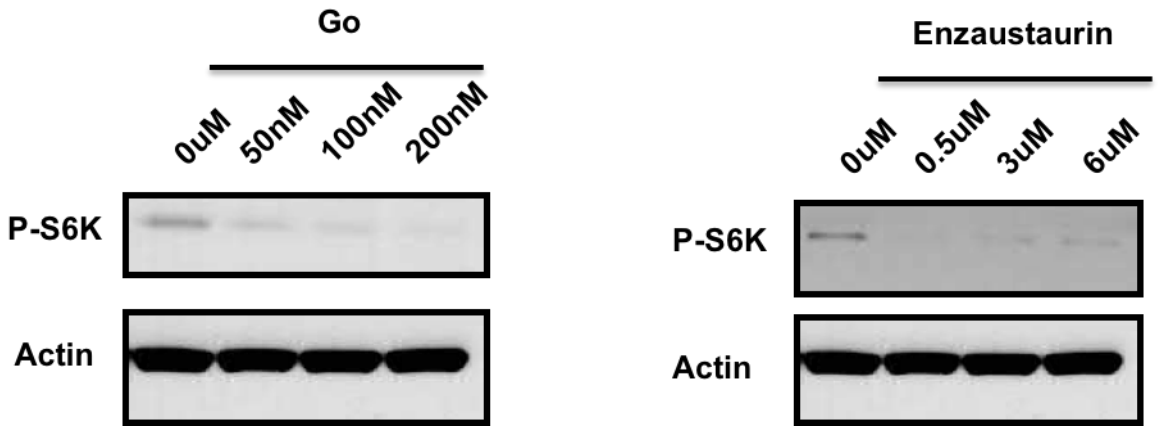
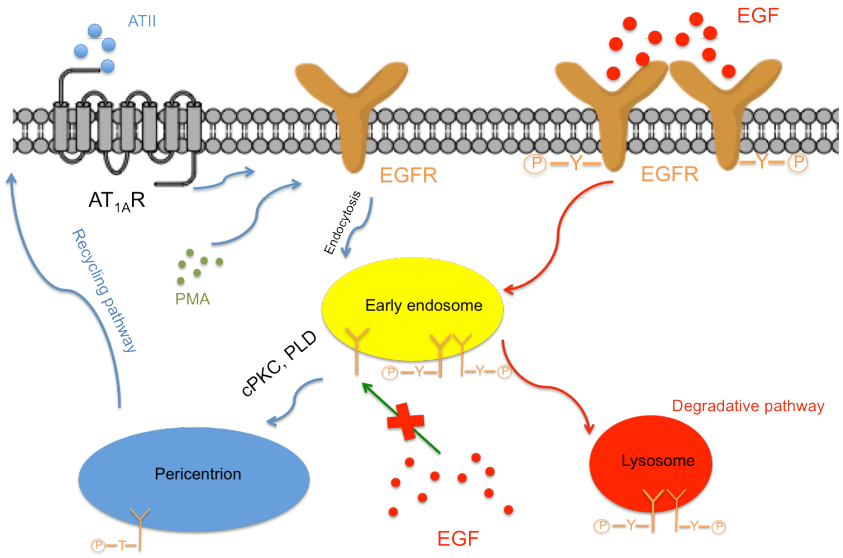
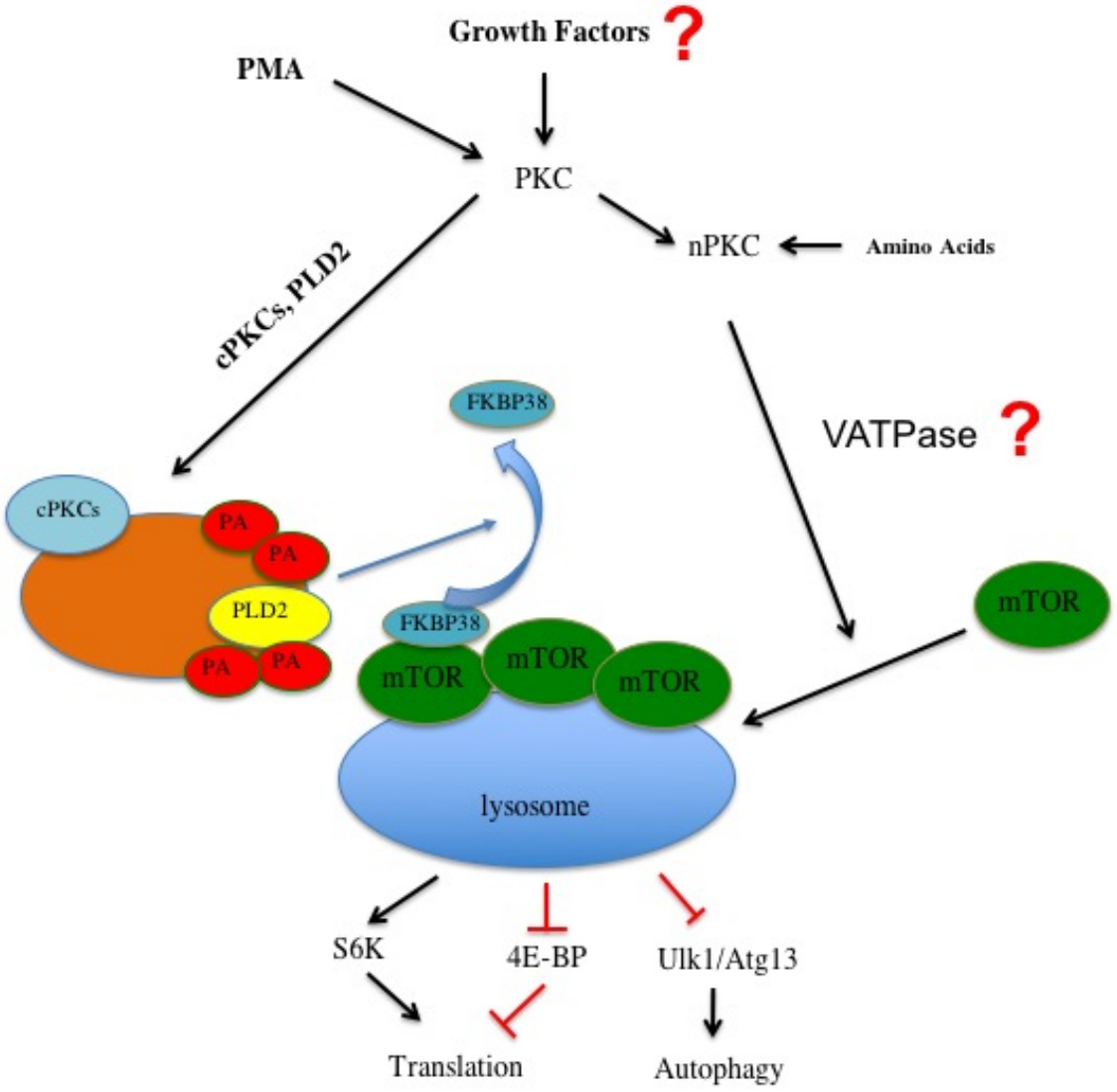


FIGURE S4. The mTORC1 activity in H292 cells requires cPKC. H4006 cells were serum starved for 5 hours and then treated with Go6975 or Enzastaurin at different concentration as indicated. Phosphorylation of S6K and actin were analyzed by western blotting.

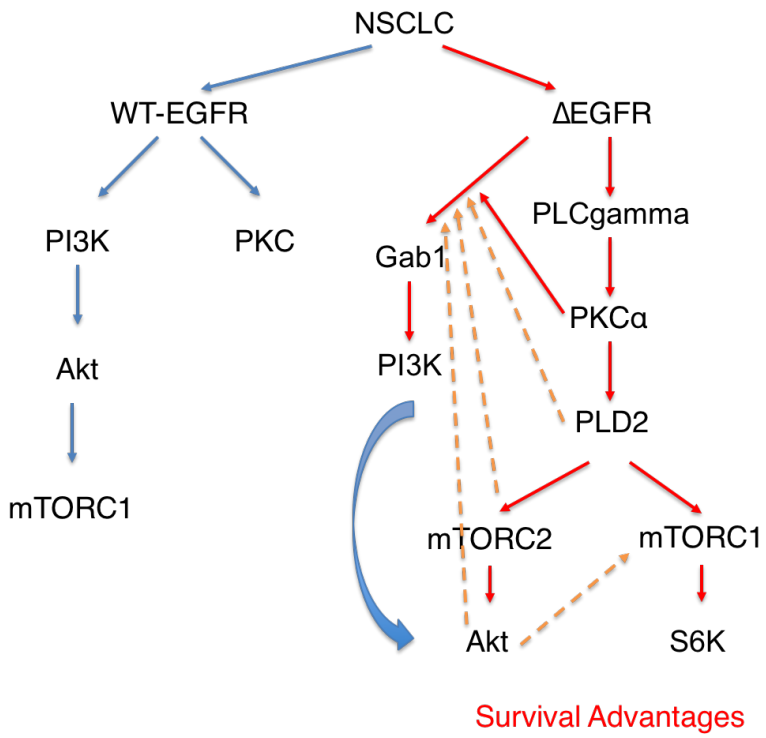
Schemes



SCHEME 1. Scheme illustrating sustained activation of PKC induces PLD- and endocytosis- dependent phosphorylation of Thr-654 on EGFR and sequestration of EGFR to a cPKC- dependent subset of recycling compartment (pericentrion). Prolonged treatment with ATII or PMA could induce translocation of EGFR to pericentrion and phosphorylation of EGFR on Thr-654 on a cPKC- and PLD- dependent manner. Sequestration of EGFR to pericentrion protects EGFR accessed by EGF.



SCHEME 2. Sustained activation of PKC activates mTORC1 through a novel and more complicated mechanism. The sustained activation of PKC induces the accumulation of mTOR in the perinuclear lysosomes and the formation of cPKC- containing endosomes. With the formation of the cPKC- containing endosomes, PLD2 and PA both accumulate in the compartment, which is in close proximity to the mTOR- containing lysosomes. Then, PA activates mTORC1 trans-compartmentally through displacing mTOR endogenous inhibitor, FKBP38.



SCHEME 3. PKCα rewires the mTORC1 and PI3K/Akt singaling in NSCLC with mtEGFR. By binding with it ligands, wild type EGFR triggers the activation of mTORC1 mainly through PI3K/Akt signaling pathway and PKC is not required for the activation of mTORC1. In NSCLC with mtEGFR, PKCα stays in the central position regulating the PI3K/Akt and mTORC1 signaling. Since mTORC2 is also involved in the activation of Akt, it is not clear that if PKC/PLD2 regulate the mTORC2 activity. Our results show that cPKC inhibitor interrupted the interaction of mtEGFR and Gab1, however it is not clear if PLD2, or mTOR2 is also involved.

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