## Growth factor signaling pathways converge on the TSC complex to control mTOR

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Growth factor signaling pathways converge on the TSC complex to control mTOR

A dissertation presented

by

Maria Erika Ilagan

to

The Committee on Higher Degrees in Biological Sciences in Public Health

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Biological Sciences in Public Health

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Growth factor signaling pathways converge on the TSC complex to control mTOR

Abstract

The mechanistic target of rapamycin complex 1 (mTORC1) serves as a molecular link between cellular growth conditions and anabolic processes that are fundamental to cell growth and proliferation. However, while it is established that the PI3K-Akt pathway regulate mTORC1 through a mechanism that involves spatial control of the TSC complex, it is unknown whether other growth factor signaling pathways do so. The goals of this dissertation were to characterize the mechanisms by which upstream growth factor signaling pathways converge on the TSC complex to activate mTORC1, and to determine the significance of these mechanisms in disease.

I identify that both the Ras-ERK and PKC pathways stimulate mTORC1 signaling through spatial regulation of the TSC complex. Growth stimulus specific to each pathway induces the dissociation of TSC2 from the lysosome, where it interacts with Rheb, and in serum-starved conditions, keeps Rheb in its GDP-bound form, unable to activate mTORC1. Using antibodies to specific TSC complex components, I further demonstrate that it is the intact TSC complex that dissociates from the lysosome upon growth stimuli treatment. Furthermore, suppression of both ERK and PKC signaling using multiple small molecule inhibitors specific to each pathway, reverses the growth stimuli-mediated TSC2 dissociation.

Given previous models on ERK-mediated TSC complex regulation of mTORC1 activity, I sought to gain more mechanistic details surrounding this regulation. I specifically show that signaling through EGF and PMA does not affect TSC complex stability, but rather regulate the
complex through effects on lysosomal localization and Rheb binding. Finally, I discuss how the TSC complex spatial regulation mechanism may be useful in predicting sensitivity or resistance of cell models to targeted therapeutics.

Collectively these studies identify a possible general mechanism by which upstream growth factor-regulated kinases activate mTORC1 through the stimulated release of the TSC complex from lysosomal Rheb. Our results advance our understanding of how diverse upstream molecular inputs can affect the physiological and oncogenic activation of mTORC1, and will hopefully guide future work in studying resistance to targeted therapeutics.
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<tr>
<td>4E-BP</td>
<td>eIF4E binding proteins</td>
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<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
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<td>AMPK</td>
<td>AMP kinase</td>
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<tr>
<td>ATF4</td>
<td>activating transcription factor 4</td>
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<tr>
<td>CAD</td>
<td>carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, dihydroorotase</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>EGF receptor</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>ERK/MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>FKBP12</td>
<td>FK506 binding protein of 12 kDa</td>
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<tr>
<td>FOXO</td>
<td>forkhead box O</td>
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<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
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<td>guanine nucleotide exchange factor</td>
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<td>HCC</td>
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<tr>
<td>HIF-1α</td>
<td>hypoxia-inducible factor alpha</td>
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<td>IGF1</td>
<td>insulin-like growth factor 1</td>
</tr>
<tr>
<td>IR</td>
<td>insulin receptor</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LAM</td>
<td>lymphangioleiomyomatosis</td>
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<tr>
<td>LC-MS/MS</td>
<td>liquid chromatography-mass spectrometry/mass spectrometry</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MEK</td>
<td>MAPK kinase</td>
</tr>
<tr>
<td>mLST8</td>
<td>mTOR-associated protein, LST8 homologue</td>
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<td>mRNA</td>
<td>messenger RNA</td>
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<td>MTHFD2</td>
<td>methylene tetrahydrofolate dehydrogenase 2</td>
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<td>mTOR</td>
<td>mechanistic target of rapamycin</td>
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<td>mTORC1</td>
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<td>phosphatidylinositol-4,5-bisphosphate</td>
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<td>PIP₃</td>
<td>phosphatidylinositol-3,4,5-triphosphate</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<td>PRAS40</td>
<td>proline-rich Akt/PKB substrate 40 kDa</td>
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<tr>
<td>PTEN</td>
<td>phosphatase and tensin homologue</td>
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<td>Rag</td>
<td>RAS-related GTP-binding protein</td>
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<td>Raptor</td>
<td>regulatory-associated protein of mTOR</td>
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<td>Rheb</td>
<td>RAS homolog enriched in brain</td>
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<td>rapamycin insensitive companion of mTOR</td>
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<td>rRNA</td>
<td>ribosomal RNA</td>
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<tr>
<td>RSK</td>
<td>MAPK-activated protein kinase-1</td>
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<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
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<td>S6</td>
<td>ribosomal protein S6</td>
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<tr>
<td>S6K</td>
<td>ribosomal S6 kinase</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>SGK</td>
<td>serum/glucocorticoid regulated kinases</td>
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<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
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<tr>
<td>mSIN1</td>
<td>mammalian stress-activated protein kinase-interacting protein 1</td>
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<tr>
<td>SREBP</td>
<td>sterol regulatory element binding protein</td>
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<td>TBC1D7</td>
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<td>UVRAG</td>
<td>UV radiation resistance-associated gene product</td>
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CHAPTER 1:
INTRODUCTION

Sections 1.1.1 and 1.2 of this chapter are adapted from:

1.1 **mTORC1 is a major regulator of cell growth and metabolism**

1.1.1 Overview

The ability of organisms to adapt to fluctuations in nutrient availability is fundamental to the survival and growth of a species. Eukaryotic cells, particularly those within multicellular organisms, have the ability to not only detect nutrients present within the cell and its surroundings, but also sense systemic signals from growth factors, cytokines, and hormones. A small number of highly connected signaling nodes are required to integrate these diverse intracellular and extracellular signals to mount an appropriate physiological response. mTORC1 sits at the convergence point of a vast signaling network that functions to integrate a wide array of signals in order to properly control cell, tissue, and organismal growth.

The evolutionarily conserved Ser/Thr kinase mTOR resides in two major protein complexes in cells, mTORC1 and mTORC2, which are distinct in their regulation, susceptibility to different classes of inhibitors, and the downstream substrates that mTOR phosphorylates as a component of each complex. Both complexes contain mTOR and a protein that associates with its kinase domain, called mLST8. It is believed that the functional differences between mTORC1 and mTORC2 stem from the other core components: Raptor for mTORC1 and a complex between Rictor and mSIN1 for mTORC2 (Fig 1.1). The activation status of mTORC1 is acutely responsive to many different signals, including both exogenous growth factors and endogenous nutrients (amino acids) and energy (ATP), and its phosphorylation of established downstream substrates is also responsive to these cues. mTORC2 activity can also be stimulated by growth factors, but it has both growth-factor responsive downstream substrates and those that are constitutively phosphorylated by mTORC2. Widely used phospho-mTOR antibodies (e.g.,
phospho-S2448) do not distinguish between mTORC1 and mTORC2, and the functional relevance of the majority of the phosphosites on mTOR remains unknown.

Upon activation, mTORC1 phosphorylates a growing number of downstream targets, including its canonical effectors ribosomal S6 kinases (S6K1 and S6K2), and eukaryotic translation initiation factor 4E (eIF4E) binding proteins (4E-BP1 and 4E-BP2). Through its downstream effectors, mTORC1 signaling promotes anabolic cell growth and inhibits the catabolic process of autophagy. In contrast, mTORC2 promotes cell survival, proliferation, and changes in the actin cytoskeleton through its phosphorylation of Akt, serum/glucocorticoid-regulated kinases (SGK), some isoforms of protein kinase C (PKC), and likely other targets.

While mTORC1 and mTORC2 have distinct downstream targets, they can phosphorylate a very similar sequence motif on the AGC family of protein kinases involved in kinase activation, referred to as the hydrophobic motif. For instance, mTORC1 phosphorylates T389 within this motif on S6K1, whereas mTORC2 phosphorylates S473 within this motif on Akt. The determinants of differential substrate specificity for these and other targets of mTORC1 and mTORC2 have not been fully elucidated, but secondary binding motifs and differential subcellular localization of the downstream targets are likely to contribute.

In addition to their unique substrate specificity, mTORC1 and mTORC2 also have differential susceptibility to the mTOR inhibitor rapamycin (Fig 1.1). Rapamycin (or sirolimus) and its analogs (e.g., everolimus, temsirolimus), collectively referred to as rapalogs, are all highly specific allosteric inhibitors of mTOR with the same mechanism of action. Rapalogs bind to the intracellular protein FKBP12, and this drug-protein complex binds to the FKBP12-rapamycin binding (FRB) domain of mTOR, which is highly conserved and lies immediately N-terminal to the kinase domain. Importantly, binding of the FKBP12-rapalog complex to the
Figure 1.1. The Two mTOR Complexes: Components, Substrates, and Inhibition

The Ser/Thr kinase mTOR exists in two structurally and functionally distinct complexes, mTORC1 and mTORC2. The core essential components of each complex are shown. mTORC1 is acutely sensitive to inhibition by rapalogs. While rapalogs do not directly inhibit mTORC2, sustained rapalog treatment can lead to dissociation and inhibition of the complex over time. Both mTORC1 and mTORC2 are equally sensitive to mTOR kinase inhibitors. mTOR phosphorylates distinct downstream substrates within mTORC1 and mTORC2, and a few of the best established targets are shown.
FRB domain only occurs when mTOR is within mTORC1 or existing as free mTOR, without additional subunits. As such, low doses of rapalogs (20 nM or less) lead to rapid inhibition (within minutes) of mTORC1 but not mTORC2.

Higher doses or prolonged exposure (several hours or days) to rapalogs can sequester free mTOR away from mTORC2 and block assembly of this complex, leading to its inhibition\(^\text{14}\). The influence of rapalogs on mTORC2 stability and activity varies greatly between different settings. While it is unknown what underlies these differences, one could speculate that the rate of mTORC2 turnover in a given setting might play a major role. Lastly, although rapalogs are highly specific inhibitors, it is now well recognized that they are only partial inhibitors of mTORC1, with many direct downstream targets being at least partially resistant to the effects of rapalogs\(^\text{15-17}\).

The sequence context of a given phosphorylation site, even distinct sites on the same downstream substrate, appears to play a particularly important role in dictating its sensitivity to rapalogs\(^\text{18}\). For instance, S6K1-T389 phosphorylation is highly sensitive to rapalogs in all settings, whereas another canonical downstream target 4E-BP1 has inhibitory sites directly phosphorylated by mTORC1 (T37 and T46) that are largely resistant. Therefore, if a cellular process is found to be resistant to rapalogs, one cannot conclude that it is occurring in an mTORC1-independent manner. Reciprocally, if something is found to be rapalog sensitive, then the effects discussed above on mTORC2 should be considered, especially when using higher doses or longer durations of rapalog treatment.

A variety of second-generation mTOR inhibitors have been developed in recent years that directly target the ATP-binding pocket of the mTOR kinase domain and, therefore, completely inhibit both mTORC1 and mTORC2 (Fig 1.1). While one cannot distinguish between effects on
mTORC1 versus mTORC2 with these inhibitors without employing additional genetic approaches, these compounds have played a critical role in uncovering functions of mTORC1 that are more resistant to rapalogs\textsuperscript{16-18}. Due to their mode of action, these compounds are not as specific as rapalogs for inhibition of mTOR. They have various off-target effects on other protein and lipid kinases, including members of the phosphoinositide-3 kinase (PI3K) family and related protein kinases, which are evolutionarily related to mTOR\textsuperscript{19}. However, reasonably selective tool compounds and clinical stage drugs have been developed. Many cancer trials are underway, or just completed, with this second generation of mTOR inhibitors, as well as those that hit both mTOR and PI3K isoforms\textsuperscript{20}.

1.1.2 Downstream processes regulated by mTORC1

Sitting at a major integration node in a vast signaling network, mTORC1 is uniquely positioned to act as a molecular link between nutrient signals and the metabolic processes required for cellular growth. In response to growth-promoting signals from exogenous growth factors and endogenous nutrients, mTORC1 is activated and promotes cellular growth and proliferation by shifting the metabolic program of the cell from catabolic (energy-producing) to anabolic (growth-promoting) metabolism (Fig 1.2). Specifically, mTORC1 stimulates a downstream program that promotes the conversion of available nutrients into biomass through the stimulation of protein, lipid, and nucleotide synthesis, processes that are all fundamental to cell growth and proliferation\textsuperscript{21-27}. Given the great demand for nutrients and energy that accompanies the mTORC1-mediated induction of these anabolic processes, the activation state of mTORC1 in normal cells is very tightly controlled.
Figure 1.2. mTORC1 links nutrient availability with the switch between catabolic and anabolic processes

Through the sensing of exogenous growth factors and intracellular nutrients and energy, mTORC1 promotes the synthesis of proteins, lipids, and nucleic acids by shifting the metabolic balance from catabolic (energy-producing) to anabolic (growth-promoting) processes. Anabolic processes can convert nutrients and energy to make macromolecules necessary for growth, while catabolic processes will break down macromolecules into their nutrient components.
Among the many downstream anabolic processes controlled by mTORC1, protein synthesis is one of the earliest identified and best-established. Through its major effectors, S6K and 4E-BP, mTORC1 activation results in an increased capacity for protein synthesis largely in part through the induction of translation of specific messenger RNAs and stimulation of ribosome biogenesis\(^28\) (Fig 1.3). Phosphorylation of 4E-BP by mTORC1 disrupts 4E-BP’s interaction with eIF4E at the 5’- cap of mRNAs, which allows the subsequent recruitment of eIF4G and assembly of the translation initiation complex\(^29\). This mechanism is particularly important for the translation of mRNAs containing 5’-terminal oligopyrimidine (TOP) and TOP-like sequences, which encode a large subset of ribosomal proteins and translation initiation and elongation factors\(^30-32\). In addition to 4E-BP, mTORC1 also phosphorylates and activates S6K1 and S6K2, which stimulate mRNA translation by phosphorylating many translation factors\(^29\). Specifically, the S6K-mediated phosphorylation of eIF4B, a binding partner of the eIF4A RNA helicase, stimulates the unwinding of mRNA secondary structure, thus promoting the translation of mRNAs with complex 5’ untranslated regions (UTRs)\(^33,34\). mTORC1 and S6K further control other aspects of mRNA translation and maturation, which includes enhancing the translation efficiency of spliced mRNAs\(^35,36\). Importantly, while the S6K-mediated phosphorylation of its namesake substrate, the S6 protein component of the 40S ribosome is widely established, the exact molecular mechanism as to how this affects translation remains elusive. Taken together, these processes lead to a global increase in mRNA translation and highlights the broad control of mTORC1 over protein synthesis.

Aside from enhanced protein synthesis, growing and proliferating cells also have an increased need for purine and pyrimidine nucleotides which make up the nucleic acids RNA and DNA. Since the majority of intracellular nucleotides reside in the ribosome, an increase in
mTORC1 stimulates downstream metabolic processes to support growth and proliferation.

When active, mTORC1 stimulates HIF1α to promote aerobic glycolysis. It also drives de novo lipid synthesis through its stimulation of SREBP processing and maturation. Via the enzyme CAD, mTORC1 drives de novo pyrimidine synthesis. It also drives de novo purine synthesis via an ATF4-dependent mechanism. Cap-dependent protein synthesis is stimulated via S6K and 4E-BP1. mTORC1 also inhibits autophagy via ULK1, and can repress lysosomal biogenesis through its phosphorylation of TFEB, causing it to be sequestered in the cytosol.
ribosome biogenesis brought about by activated mTORC1 signaling results in an overall greater demand for nucleotides. Nucleotides can be acquired through either de novo synthesis or exogenous uptake pathways. Recent studies have shown that through a variety of transcriptional and post-translational mechanisms, mTORC1 stimulates metabolic flux through the de novo pyrimidine and purine synthesis pathways\textsuperscript{21,22}. The regulation of de novo pyrimidine synthesis occurs through S6K-mediated phosphorylation of CAD (carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, dihydroorotase), the rate-limiting enzyme that catalyzes the first three steps of de novo pyrimidine synthesis\textsuperscript{21,22} (Fig 1.3). While S6K-mediated phosphorylation of CAD is not necessary to basal activity of de novo pyrimidine synthesis, it does increase the pathway activity in response to stimuli that activate mTORC1. In addition, mTORC1 signaling also promotes de novo purine synthesis, albeit with delayed kinetics, through the regulation of transcription factors Myc, SREBP (sterol regulatory element binding protein) and ATF4 (activating transcription factor 4), which induce the expression of key metabolic enzymes in this pathway, as well as pathways that supply essential precursors, including the pentose phosphate, serine synthesis, and mitochondrial tetrahydrofolate (mTHF) pathways\textsuperscript{26}. For instance, mTORC1 activation of ATF4 induces an increase in MTHFD2 (methylene tetrahydrofolate dehydrogenase 2) expression, a key enzyme in the mTHF cycle which provides cytosolic one-carbon units required for purine ring assembly\textsuperscript{26}. Collectively, these pathways that are influenced by mTORC1 activity coordinate to ensure that the high demand for nucleotides necessary for downstream anabolic processes is sufficiently met.

Along with proteins and nucleotides, proliferating cells also have an increased demand for lipids, which are required for a variety of processes including incorporation into organelles and the plasma membrane. mTORC1 has emerged as a major promoter of de novo lipid synthesis
through activation of the SREBP family of transcription factors\textsuperscript{37,38}. The SREBPs (SREBP1a, SREBP1c, and SREBP2) are master regulators of genes encoding most lipogenic enzymes involved in the synthesis of both fatty acids and sterols\textsuperscript{38-41}. Furthermore, SREBPs also provide reducing power for \textit{de novo} lipid synthesis through transcriptional activation of NADPH-producing enzymes\textsuperscript{42}. While the exact molecular mechanism is unknown, mTORC1 stimulates the maturation and subsequent activation of SREBPs through at least two mechanisms, one involving S6K1\textsuperscript{42}, and another involving the mTORC1-mediated inhibition of Lipin1, an inhibitor of SREBP\textsuperscript{43}. Other downstream targets of mTORC1 may also affect SREBP processing and activity, and may do so in a context specific-manner\textsuperscript{27,44}. Finally, the induction of \textit{de novo} lipid synthesis downstream of mTORC1 has been established in a variety of settings, both in cultured cells and the liver, highlighting the crucial role of mTORC1 in regulating this macromolecule\textsuperscript{37,42-46}.

Aside from its central role in protein, nucleotide, and lipid synthesis, mTORC1 signaling also stimulates other metabolic processes to further support its anabolic program. Specifically, mTORC1 promotes a shift from oxidative phosphorylation to aerobic glycolysis, (a process known as the Warburg effect) by increasing expression of the glucose transporter GLUT1, and glycolytic enzymes that are encoded by gene targets of HIF1α (hypoxia-induxible factor 1α)\textsuperscript{42} (Fig 1.3). Similar to HIF1α, Myc can also upregulate expression of genes involved in glucose uptake and glycolysis in an mTORC1-dependent manner\textsuperscript{47}. The overall shift towards glycolysis supports anabolic metabolism by allowing the cell to generate metabolic intermediates that can be used in key side branches, such as the pentose-phosphate and serine/glycine synthesis pathways\textsuperscript{28}. These pathways in turn provide required precursors used in biosynthetic processes for cell proliferation.
Parallel to its role in promoting macromolecular synthesis necessary for cell growth and proliferation, mTORC1 suppresses the breakdown of macromolecules by inhibiting catabolic processes such as autophagy and lysosome biogenesis (Fig 1.3). When autophagy is active, cells recycle their cytosolic proteins and organelles into their nutrient components through engulfment by the autophagosome, and eventual fusion with the lysosome where degradation occurs\textsuperscript{48}. The best characterized mechanism by which mTORC1 suppresses autophagy is through the mTORC1-mediated phosphorylation and inhibition of the protein ULK1 (Unc51-like kinase 1), which is a key regulator of autophagy induction, specifically the early stages of autophagosome formation\textsuperscript{49-52}. More recent work has also implicated mTORC1’s role in inhibiting later stages of autophagy by phosphorylating UVRAG (UV radiation resistance-associated gene product), an essential component of the autophagosome and endosome maturation process\textsuperscript{53}. Beyond autophagy, there has also been increasing evidence for mTORC1’s influence on overall lysosomal health and function. Specifically, inhibition of mTORC1 signaling promotes a transcriptional program that induces the expression of genes encoding the autophagy machinery and components of the lysosome\textsuperscript{54-57}. When active, mTORC1 phosphorylates TFEB (transcription factor EB) causing it to be sequestered in the cytosol and unable to initiate its gene transcriptional program. The consequences of this direct phosphorylation of TFEB by mTORC1 could contribute to the long-term inhibitory effect of mTORC1 signaling on autophagy and lysosome homeostasis. Finally, the coordinated regulation of lysosomal health with the mTORC1 signaling network is yet another example of how cells actively adapt to fluctuations in nutrient availability within their environment.
1.1.3 Upstream regulation of mTORC1 signaling

The integrated control of mTORC1 involves two systems of small G proteins, the Rag and Rheb GTPases, which lie upstream and directly engage the kinase complex. The Ras-related small G protein Rheb (Ras homolog enriched in brain), upon GTP loading, becomes an essential and potent upstream activator of mTORC1, and appears to be necessary for activation by all signals. Until recently, the exact molecular mechanism as to how Rheb activates mTORC1 has been largely unknown. Recent insights from Cryo-EM structural studies show that Rheb-GTP directly binds mTOR within mTORC1, and after a series of conformational changes induced by this binding, the kinase becomes activated. Importantly, a subpopulation of Rheb resides on the cytosolic surface of the lysosome, where it’s held partly through farnesylation, and where it encounters mTORC1.

The Rag (Ras-related GTP binding protein) GTPases function to recruit mTORC1 to lysosomal Rheb. The Rag proteins consist of four family members, and are obligate heterodimers comprised of either RagA or RagB with RagC or RagD. These heterodimers are tethered to the lysosomal surface through interactions with the Ragulator complex (LAMTOR), which has guanine-nucleotide exchange factor (GEF) activity toward RagA/B, and the lysosomal amino acid transporter, SLC38A9 (Fig 1.4). When sufficient amino acids are available, the Rags are converted to their active state (RagA/B is GTP-loaded, while RagC/D is GDP-loaded) and can bind the Raptor subunit of mTORC1. This direct binding results in recruitment of mTORC1 to the lysosomal surface, where it is then primed to be activated by Rheb, when Rheb is GTP-bound. Thus, the recruitment of mTORC1 to the lysosomes by the Rags is necessary, but not sufficient for its activation. Conversely, while Rheb is able to activate mTORC1, it is not sufficient for its lysosomal recruitment. The coordinated actions of the Rag
Figure 1.4. Amino acid sensing at the lysosome  (From Valvezan and Manning, 2019)  

Under conditions of sufficient amino acids, mTORC1 is recruited to the lysosomal surface via the Rag proteins and an upstream amino acid sensing pathway involving GATOR1 and 2, Sestrin, and CASTOR1. mTORC1 is also sensitive to intra-lysosomal amino acids via the V-ATPase, which interacts with the Ragulator complex to stimulate its GEF activity toward RagA/B.
proteins, downstream of amino acids, and Rheb, downstream of growth factors, for regulation of mTORC1 on the surface of lysosomes, highlight the dynamic signal integration capabilities of the mTORC1 network.

Once at the lysosome, mTORC1 is sensitive to both cytosolic and intra-lysosomal amino acid levels. mTORC1 senses lysosomal amino acids through a mechanism that requires the V-ATPase, which interacts with the Rag and Ragulator complex to promote RagA/B activation. Rag protein activity is further regulated by the upstream protein complex GATOR1 (comprised of DEPDC5, Nprl2, and Nprl3), which acts as a GAP (GTPase-activation protein) for RagA/B, and associates with the lysosome through the lysosome resident protein complex KICSTOR (comprised of Kaptin, ITFG2, C12orf66, and SZT2). In the absence of amino acids, GATOR1 is active, which promotes the accumulation of GDP-bound RagA/B, thereby blocking the recruitment of mTORC1 to the lysosome. GATOR1 is itself inhibited by the mTORC1-activating complex GATOR2 (comprised of Mios, WDR24, WDR59, Seh1L, and Sec13), which is sensitive to both cytosolic leucine and arginine levels through Sestrin2 and CASTOR1 (cellular arginine sensor for mTORC1) respectively (Fig 1.4). Another mechanism that contributes to the conversion of RagA/B to the GTP-bound active state, and consequently influence the activation of mTORC1, include the GEF (guanine exchange factor) activity of the lysosomal amino acid transporter SLC38A9 towards RagA/B. Finally, identification of the folliculin-FNIP2 complex as a GAP for RagC/D at the lysosomal surface, is yet another mechanism to add to this intricate pathway upstream of mTORC1 dedicated to amino acid sensing.

Similar to the upstream amino acid sensing pathways, signals from growth factors and hormones that are upstream of mTORC1 are also sensed and coordinated at the lysosome. These
pathways converge on the TSC protein complex, a major upstream regulator of mTORC1\textsuperscript{80}. The TSC complex is a heterotrimeric protein complex composed of the tuberous sclerosis complex (TSC) tumor suppressors TSC1 and TSC2, and a third regulatory subunit TBC1D7 (Tre2-Bub2-Cdc16 (TBC) 1 domain family member 7)\textsuperscript{81,82}. Unlike the previously discussed Rag proteins, the only well-established regulator of Rheb is the TSC complex. The TSC2 subunit of the TSC complex possesses GAP activity towards Rheb, which promotes the conversion of Rheb to its GDP-bound state, incapable of activating mTORC1\textsuperscript{60,81,83-86}.

The TSC complex is acutely regulated by at least two of the major growth factor signaling pathways in cells, the PI3K-Akt and Ras-ERK pathways (Fig 1.5). The PI3K-Akt pathway is stimulated by binding of growth-promoting molecules such as insulin or IGF1 (insulin-like growth factor 1) to cell surface receptor tyrosine kinases (RTKs), which stimulates PI3K (phosphoinositide 3-kinase) activity\textsuperscript{87}. PI3K is a lipid kinase that phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP\textsubscript{2}) to produce phosphatidylinositol-3,4,5-triphosphate (PIP\textsubscript{3}). Conversely, the pathway can be repressed by the conversion of PIP\textsubscript{3} back to PIP\textsubscript{2} through the actions of PTEN (phosphatase and tensin homolog), a frequently disrupted tumor suppressor. PIP3 acts as a lipid second messenger and recruits Akt to the plasma membrane, where it is phosphorylated by membrane-bound phosphoinositide-dependent kinase (PDK1) at T308\textsuperscript{88}. This phosphorylation event only partially activates Akt, and a second phosphorylation event at S473 by mTORC2\textsuperscript{4} is required for maximal activation\textsuperscript{89}. Once activated, Akt regulates many biological processes through its phosphorylation of proteins containing an RXXS/T motif\textsuperscript{90}. The primary route of Akt-mediated activation of mTORC1 is through its multi-site phosphorylation of TSC2, which leads to the functional inhibition of the TSC complex\textsuperscript{91,92}. Specifically, this
Figure 1.5. The PI3K-Akt and Ras-ERK pathways regulate the TSC complex

The PI3K-Akt pathway (stimulated by insulin and IGF) and the Ras-Erk pathway (stimulated by EGF) converge on the TSC complex to regulate mTORC1 signaling. The presence of growth stimuli inactivate the TSC complex, keeping Rheb in its GTP bound form and activating mTORC1 as a result.
phosphorylation causes the TSC complex to dissociate from the lysosomal membrane, allowing GTP-bound Rheb to accumulate and activate mTORC1 at the lysosome\textsuperscript{60}.

Aside from PI3K-Akt, the Ras-ERK pathway also regulates mTORC1 through the TSC complex. Growth factors such as epidermal growth factor (EGF) bind to cell-surface RTKs which then leads to receptor auto-phosphorylation and subsequent recruitment and stimulation of the Ras GTPase\textsuperscript{93}. Ras then recruits Raf to the plasma membrane where it is activated. Activated Raf phosphorylates MEK, a mitogen-activated protein kinase (MAPK) kinase, which in turn phosphorylates ERK (extracellular signal-regulated kinase or MAPK)\textsuperscript{94}. ERK is a major effector of the Ras pathway and targets many substrates that regulate cell cycle progression and growth. One of the cytoplasmic signaling proteins that ERK phosphorylates is p90 ribosomal S6 kinase (RSK), which in turn also phosphorylates other targets and regulators\textsuperscript{94}. Similar to Akt, ERK and RSK also regulate mTORC1 activity through site-specific inhibitory phosphorylation events on TSC2\textsuperscript{91,92,95-97}. The convergence of two of the most major growth signaling pathways, PI3K-Akt and Ras-ERK, on the TSC complex highlights the profound significance of this signaling node to multiple disease settings.

Beyond PI3K-Akt and Ras-ERK, other important pathways also impinge on regulation of the TSC complex and Rheb. This includes the AMPK pathway, which has evolved as the major sensor of energy stress in the cell. During conditions of energy depletion, high cellular AMP levels allosterically activate AMPK (AMP-activated protein kinase) to respond to cellular energy stress. AMPK then phosphorylates distinct sites on TSC2, activating the TSC complex and promoting its ability to inhibit Rheb-mTORC1 signaling, thereby ensuring that mTORC1 activity can be turned off in energy-stressed conditions\textsuperscript{98-100}. Lastly, the Wnt signaling cascade, a major
upstream growth and proliferation pathway, can also regulate mTORC1 activity via GSK3 (glycogen synthase kinase 3)-mediated phosphorylation of TSC2.

1.1.4 Integrated regulation of mTORC1 by nutrients and growth factors at the lysosome

As a master regulator of metabolic processes crucial to growth and proliferation, it is not surprising that the activation state of mTORC1 is tightly regulated. The downstream anabolic processes that mTORC1 influences are very costly in terms of carbon and ATP. Thus, cells have evolved sophisticated mechanisms by which the intracellular availability of nutrients and energy influence the activation state of mTORC1. Specifically, the availability of building blocks and precursors (e.g. amino acids, nucleotides, etc.) for the macromolecules that they make up, strongly influence mTORC1 activity. Importantly, depletion of any individual intracellular nutrient renders mTORC1 unable to kickstart its entire downstream anabolic program, thus putting a brake on macromolecular synthesis.

Although nutrients are required for mTORC1 signaling, it is important to note that nutrients are only sufficient for low basal mTORC1 activation. While basal activation does inhibit autophagy, at this state, mTORC1 is not fully activated to stimulate its entire anabolic program. In addition to nutrients, extracellular signals such as growth factors, hormones, and cytokines are required for full activation of the kinase complex. Conversely, the availability of growth factors but depletion of nutrients fail to activate mTORC1 signaling. Thus, intracellular nutrients are required for mTORC1 signaling, but not sufficient for full activation. This complex integration of signals, both from nutrients and growth factors, to activate mTORC1 is thought to be partly influenced by molecular events that occur on the surface of the lysosome.
1.2 **The central role of mTOR in the response to cancer therapeutics**

1.2.1 Overview

Cancer cells exhibit cell autonomous growth and proliferation that is largely disconnected from exogenous growth factors. The genetic events underlying this property lead to constitutive activation of mTORC1 in the majority of human cancers, across nearly all lineages. Upstream activating mutations or amplifications in common oncogenes, such as RTKs, oncogenic fusion proteins, PI3K, AKT, RAS, and RAF, and loss of function mutations in common tumor suppressors, all lead to aberrant activation of mTORC1 (Fig1.6). The convergence of these oncogenic signaling pathways on mTORC1 and the large number of distinct genetic alterations that can lead to uncontrolled mTORC1 signaling in tumors, together with its key role in anabolic tumor growth, make mTORC1 both a promising target for cancer therapy and a major mechanism of resistance to targeted therapeutics.

1.2.2 mTOR inhibitors as single agent therapeutics

Due to the prevalence of mTORC1 activation in human cancers, there has been a long-time interest in mTORC1 inhibitors for the treatment of a wide variety of cancers, including solid carcinomas and sarcomas, as well as those of hematopoietic origins. These inhibitors include rapamycin and its analogs (rapalogs), which act through allosteric mechanisms, and more recently, mTOR kinase domain inhibitors. While we await the outcome of trials using mTOR kinase inhibitors, rapalogs have already been tested in nearly one thousand clinical cancer trials, across most cancer lineages and genetic tumor syndromes (http://clinicaltrials.gov). With few exceptions, the results of single-agent rapalog trials suggest that inhibiting mTORC1 alone is not sufficient to cause tumor regression in the majority of cases. A notable exception is results from
Figure 1.6. A network of oncogenes and tumor suppressors converges on the regulation of mTORC1

Some of the most common oncogenes (depicted in green), including receptor tyrosine kinases (RTKs), oncogenic fusion proteins formed by chromosomal translocations, PI3K, Akt, Ras and Raf, and tumor suppressors (depicted in red), including PTEN, NF1, and LKB1, lie within signaling pathways that share mTORC1 regulation as a downstream target. In normal cells, growth factors bind to and stimulate RTKs and G protein-coupled receptors (GPCR), which can activate mTORC1 through either the PI3K-Akt or Ras-Erk signaling pathways. These pathways...
converge to inhibit the TSC complex (TSC1, TSC2, and TBC1D7), resulting in the accumulation of GTP bound Rheb, which is a potent and essential activator of mTORC1. Other pathways including LKB1-AMPK and Wnt signaling also impinge on regulation of the TSC complex and Rheb. A parallel pathway exists for amino acid sensing that involves the Rag family of GTPases, which act as a heterodimer of RagA or B with RagC or D, and are regulated by different upstream sensors of amino acids. Oncogenic mutations within this network are common in cancer, resulting in aberrant activation of mTORC1 signaling and its promotion of anabolic processes underlying cell growth and proliferation.

Figure 1.6. (Continued).
trials on TSC and a related lung disorder, lymphangioleiomyomatosis (LAM), which occurs in TSC patients or sporadically through mutations in TSC2. Due to their central molecular function in inhibiting mTORC1, loss of the TSC tumor suppressors leads to robust constitutive activation of mTORC1 in TSC and LAM lesions. The dependence of tumor growth on mTORC1 in these settings is evident, as rapalogs shrink tumors by approximately 50% and improve clinical outcomes \(^{105-107}\). Interestingly, within cancer trials with rapalogs, extraordinary responders have been reported, and there is some genetic evidence that such responses might be dictated by tumor-specific loss of function mutations in TSC1 or TSC2 (e.g., Ref. \(^{108,109}\)). Tumors with rare activating mutations in mTOR, itself, have also been found to exhibit exceptional sensitivity to rapalogs \(^{110,111}\). Reciprocally, a mutation in the FRB domain of mTOR was found to underlie acquired resistance to rapalogs in a case of anaplastic thyroid cancer that had a TSC2 mutation and exhibited an exquisite response initially \(^{109}\). It will be important to determine if such mutations arise in the tumors of TSC and LAM patients, who face prolonged use of rapalogs.

Given the key role for mTORC1 in driving anabolic metabolism in cancer cells, it is somewhat surprising that rapalogs are not more effective on their own. There are likely several contributing factors to their limited success. First among these is that rapalogs are only partial inhibitors of mTORC1. Furthermore, rapalogs have been found to elicit cytostatic rather than cytotoxic responses, arresting or delaying cells in the G1 phase of the cell cycle in eukaryotic cells from yeast to human \(^{112}\). Even the highly responsive tumors from TSC and LAM patients rapidly regrow upon removal of these drugs \(^{105,106}\), suggesting that the tumors shrink due to cytostatic and cell size effects rather than tumor cell death. Rapalogs have a particularly strong anti-proliferative effect on lymphocytes, which underlies their common use as immunosuppressant agents \(^{113}\). These immunosuppressive properties could provide an additional
mechanism, not intrinsic to the tumor cells, that limits the anti-tumor effects of raplogs by hindering immune clearance of tumor cells in the tumor microenvironment. The molecular wiring of the mTOR signaling network might further prevent cytotoxic responses from rapalogs. The inhibition of mTORC1 relieves a large number of distinct negative feedback mechanisms that normally serve to attenuate upstream RTK, PI3K, and mTORC2 signaling, thereby promoting the activation of downstream cell survival pathways\textsuperscript{114-122}. Finally, as discussed above, mTORC1 is most frequently activated in cancers through oncogenic events lying upstream (Fig 1.6). While mTORC1 is a shared downstream effector, it represents just one branch of the highly branched oncogenic signaling nodes most commonly activated in cancer, including RTKs, RAS, and PI3K. As such, mTORC1 inhibition alone is not sufficient to overcome the entire oncogenic program propagated from these pathways. However, accumulating evidence indicates that efficient mTORC1 inhibition might be essential for a given treatment to promote tumor regression.

1.2.2 Sustained mTORC1 signaling as a driver of resistance to targeted therapeutics

Multiple studies in distinct cancer lineages have revealed that mTORC1 inhibition, while not sufficient in most cases, is likely necessary to achieve a clinical response to drugs that target the primary oncogenic pathway in a given cancer. Using genetically-defined cancer cell lines, tumor models and patient samples, these studies have all arrived at the same conclusion: sustained mTORC1 signaling following treatment with a targeted therapeutic is strongly associated with both innate and acquired resistance to that therapeutic\textsuperscript{123}. Thus, while mTORC1 inhibitors appear to be limited in their use as single agent therapies, there is growing evidence that they may be effective in combinatorial approaches to cancer treatment, specifically when
used with targeted therapeutics. Below are three distinct cancer settings as examples where the role of mTORC1 in therapeutic resistance has been defined.

**EGFR-mutant lung cancer**

The epidermal growth factor receptor (EGFR) is an RTK important for many developmental and physiological processes, and it is frequently activated, by mutation or amplification, in a subset of human cancers\(^1\). Ligand binding to EGFR induces activation and downstream stimulation of signaling pathways, including the RAS-RAF-MEK-ERK and PI3K-Akt pathways. It is through these pathways that improper activation of EGFR in cancer promotes tumor development progression\(^2\). Activating mutations in *EGFR*, which cluster in its kinase domain, occur in up to 15% of human non-small cell lung cancer (NSCLC). In-frame deletions in exon 19 and the L858R mutation account for 85% of all oncogenic *EGFR* mutations and confer sensitivity to the tyrosine kinase inhibitors (TKIs) erlotinib and gefitinib\(^3\). However, despite multiple clinical trials showing robust initial response rates of *EGFR*-mutant tumors to TKIs, the emergence of resistance is prevalent\(^4\). The most common mechanism of resistance in lung cancer is associated with the acquisition of a secondary mutation in EGFR itself (T790M), rendering the activated kinase insensitive to these small molecule inhibitors\(^5,6\). As such, second and third generation small molecule inhibitors and therapeutic antibodies that at least partially overcome this resistance at the level of EGFR have been developed, and include HKI-272 (neratinib), afatinib, and cetuximab\(^7\). However, activation of bypass signaling events through additional oncogenic mutations, for instance in other RTKs, leading to sustained Ras or PI3K signaling has emerged as a major mechanism of resistance to this second generation of EGFR inhibitors in EGFR-mutant NSCLC\(^8,9,10\).
Multiple independent studies in cell and mouse models of EGFR-mutant NSCLC have demonstrated that sustained, or incompletely inhibited, mTORC1 signaling can contribute to TKI resistance. Using a genetically engineered mouse model (GEMM) of lung cancer driven by \( EGFR^{L858R+T790M} \), it was found that a combination of HKI-272 and rapamycin was greatly superior to either drug alone in causing tumor regression \(^{132}\). The combination of afatinib and cetuximab has been found to overcome resistance to first generation TKIs in \( EGFR^{L858R+T790M} \)-induced lung cancer \(^{133}\). However, clinical trials using this drug combination showed a less than 30% patient response rate, with median response duration of less than 6 months \(^{134,135}\). As observed in the human tumors, resistance to combined treatment with afatinib and cetuximab in both xenograft and \( EGFR^{L858R+T790M} \) GEMM tumors was associated with sustained mTORC1 signaling, and rapamycin could overcome this resistance \(^{136}\). Interestingly, sequencing of biopsies from resistant tumors revealed a frameshift mutation in TSC1 in one of the tumors, suggesting loss of function of the TSC complex leading to constitutive mTORC1 signaling. Knockdown of \( TSC1 \) expression induced afatinib resistance in an otherwise sensitive \( EGFR^{L858R+T790M} \) NSCLC line, providing genetic evidence that sustained mTORC1 signaling is sufficient to cause resistance. An additional study found that rapamycin prevents lung tumor formation in an inducible \( EGFR^{L858R+T790M} \) GEMM and can increase progression-free survival after treatment with an EGFR TKI \(^{137}\). Taken together, these studies suggest that sustained mTORC1 signaling underlies the acquired resistance of EGFR-mutant NSCLC to treatments that effectively target EGFR.

**PIK3CA-mutant breast cancer**

It has been estimated that mutations leading to constitutive activation of the PI3K-Akt pathway occur in >70% of breast cancers \(^{138}\), and activating mutations in \( PIK3CA \), encoding the
p110α isoform of class I PI3K, are particularly prevalent, accounting for ~30% of cases \(^{139}\). Drugs that selectively target the p110α isoform (e.g., BYL719) or multiple isoforms of class I PI3K (e.g., GDC0941) are in clinical development \(^{140}\), with some clinical trials using PIK3CA mutations to strictly stratify the patient population \(^{141,142}\). While this patient stratification clearly increases response rates in such trials, non-responders are still common, indicating innate resistance of these tumors. A similar phenomenon has been observed in a large panel of breast cancer cell lines, where PIK3CA mutations are the best predictor of sensitivity to BYL719 but strongly resistant cell lines with these mutations also exist \(^{143}\). While PI3K inhibitors properly block PI3K-Akt signaling in the resistant lines, these cells invariably exhibited sustained mTORC1 signaling. Furthermore, when BYL719-sensitive cell lines were selected for resistance, the resistant derivatives had reactivated mTORC1. Treatment with a rapalog was sufficient to sensitize the resistant cells to BYL719, and the combined treatment halted xenograft tumor growth, whereas either treatment alone had little to no effect \(^{143}\). Importantly, the status of a commonly used marker of mTORC1 signaling, phosphorylation of the ribosomal protein S6 (phospho-S6), appears to predict sensitivity or resistance in biopsies from breast cancer patients treated with BYL719. PIK3CA-mutant tumors that respond show inhibition of phospho-S6, whereas those with either innate or acquired resistance show continued presence of phospho-S6 with BYL719 treatment. Therefore, sustained mTORC1 signaling in PIK3CA mutant breast cancers treated with PI3K inhibitors is strongly associated with both innate and acquired resistance.

**BRAF-mutant melanoma**

The BRAF oncogene is a Ser/Thr kinase that is activated by somatic point mutations, most commonly V600E, resulting in constitutive activation of the RAF-MEK-ERK signaling
pathway in a subset of human cancers\textsuperscript{144-146}. B-RAF\textsuperscript{V600E} is the driver oncogene in over 50% of melanomas. There have been excellent advances in the development of RAF and MEK inhibitors to target this pathway, with FDA-approved drugs for treating metastatic \textit{BRAF}-mutant melanoma including the MEK inhibitor trametinib and two RAF inhibitors, vemurafenib and dabrafenib\textsuperscript{147-149}. However, despite robust initial responses to these agents being frequently observed, drug resistance eventually emerges, dampening the long-term clinical effects of these treatments\textsuperscript{145,146}. Accordingly, various mechanisms of resistance to \textit{BRAF}-targeted therapies have been investigated\textsuperscript{145,146}, with some studies pointing to a role for the establishment of alternative routes to mTORC1 activation\textsuperscript{150,151}. In a panel of melanoma cell lines, the responsiveness of phospho-S6, rather than the phosphorylation of ERK, corresponded to the sensitivity or resistance to RAF or MEK inhibitors\textsuperscript{150}. The detection of either inhibited or sustained phospho-S6 also tracked with respective sensitivity or resistance to RAF-MEK pathway inhibitors in xenograft tumor models and patient biopsies. In the melanoma patients, sustained phospho-S6 was associated with poor prognosis, and when initial responders to the treatment progressed, subsequent biopsies showed reappearance of phospho-S6. Thus, mTORC1 activation through a RAF-MEK-independent mechanism contributes to both innate and acquired resistance in melanoma. As seen in another study using a rapalog in a genetic mouse tumor model of resistance in \textit{BRAF}-mutant melanoma\textsuperscript{151}, mTOR kinase inhibitors greatly enhanced the response of resistant cell lines to RAF-MEK inhibitors\textsuperscript{150}. These studies provide evidence that mTORC1 inhibition is necessary for effective tumor response to RAF-MEK-targeted therapeutics in melanoma.
1.2.3 A model of resistance and strategies to prevent or overcome resistance

What has emerged in multiple distinct cancer lineages is that, while mTORC1 inhibition alone may not be sufficient, it is likely necessary to achieve a clinical response to drugs targeting the primary oncogenes in a given cancer (Fig 1.7). Given the diverse settings discussed above, it seems likely that sustained mTORC1 signaling will be found as a major mechanism of resistance in many cancers. For instance, it was recently shown that activation of an alternate route to mTORC1 underlies the resistance of PIK3CA-mutant head and neck squamous cell carcinomas to a PI3K-p110α inhibitor\(^\text{152}\). Identifying the most common, lineage-specific mechanisms leading to sustained mTORC1 signaling that are independent of the original therapeutic target is key to developing strategies for more effective combination therapies. A common mechanism will likely be through the upregulation, amplification, or oncogenic activation of upstream RTKs\(^\text{152,153}\). Also, oncogenic activation of the PI3K-Akt pathway in Ras or Raf-driven cancers, and vice-versa, will maintain mTORC1 signaling in the face of treatments that target these common oncogenic pathways. While TSC mutations do occur in sporadic cancers, such as bladder cancer, they are rare in most cancers (<5%). However, given its central role in mTORC1 regulation, it seems likely that more mutations affecting the function of the TSC complex will be identified in future genetic studies aimed at revealing the mode of innate or acquired resistance in different cancer settings. In the three cancers discussed above, loss of function of the TSC complex was shown to confer resistance to targeted therapeutics in cell-based models\(^\text{136,143,150}\). While mutations leading to increased signaling of the Rag GTPases to mTORC1, or activating mutations on mTOR itself, have been identified\(^\text{110,111,154,155}\), the vast majority of oncogenes and tumor suppressors influencing the activation state of mTORC1 go through the TSC complex (Fig 1.6). Therefore, it is likely that, whether it be through direct
Figure 1.7. The role of sustained mTORC1 signaling in resistance to targeted therapeutics

A simple model of the response and progression of a tumor treated with a therapeutic targeting the initial driving oncogenic pathway. Inhibitor A blocks Oncogene A (Onc-A), which attenuates signaling to multiple downstream effectors, including mTORC1. If the Onc-A pathway is the primary, non-redundant route to mTORC1, a robust anti-tumor response will be observed. Acquired resistance can develop due to bypass activation of a parallel pathway through Oncogene-B (Onc-B) resulting in sustained mTORC1 signaling in the presence of Inhibitor-A. This resistance can be overcome through combination therapy with an mTOR inhibitor in settings of both innate (i.e. initial non-responsive) and acquired resistance.
mutation or genetic events affecting upstream components of the network, aberrant inhibition of the TSC complex will frequently underlie sustained mTORC1 signaling, and thus, therapeutic resistance.

The collective findings from the studies discussed above suggest a new paradigm in precision medicine to enhance and prolong tumor responses to targeted therapeutics. Patient stratification based on the identification of driver oncogenes remains a priority for selecting the primary molecular target and corresponding inhibitor. Where possible, tumor biopsies before treatment and again early on in the treatment, with assessment of markers of mTORC1 signaling, such as phospho-S6, would be powerful in predicting responders and non-responders. Those with sustained mTORC1 signaling could immediately move to combination therapy with an mTOR inhibitor. However, in therapeutic settings where most, if not all, patients eventually become refractory to the initial treatment, perhaps combined treatment with an mTOR inhibitor from the start would prevent or delay the development of resistance. The potential toxicity of such combinations, especially with the newer mTOR kinase inhibitors, will be a major consideration.

The past 20 years has seen tremendous progress in identifying the oncogenes and tumor suppressors that provide the genetic basis of cancer development. This knowledge and a molecular understanding of the pathways influenced by these genetic changes has allowed a movement toward treating cancer in a more personalized manner. However, the use of targeted therapeutics to treat cancer has also uncovered a tremendous plasticity to this disease and the underlying oncogenic network that make resistance through pathway rewiring inevitable. The emergence of mTORC1, a highly connected node within this network, as a major contributor to both innate and acquired resistance provides a key secondary target to which there already exists
specific pharmacological agents. Understanding network rewiring and resistance at the molecular level is essential to developing more effective treatments for cancer.

1.3 Overview of the Dissertation

Genomic analysis of tumors combined with the rational development of drugs targeting common oncogenic drivers have paved the way for personalized and precision medicine approaches to cancer therapy. While patient stratification based on tumor genotype improves the effectiveness of cancer therapeutics, in most cases, non-responders are still common, indicating an innate resistance of these tumors. Signal rewiring and redundancy within oncogenic signaling networks can underlie resistance of tumor cells to targeted therapeutics. While a great deal of work has been done in the last two decades to study growth signaling pathways, the inherent complexity of signaling networks, with its many branches and points of convergence, has made it difficult to gain a complete picture. In this dissertation, I characterize the molecular mechanisms by which the two major growth factor signaling and oncogenic pathways in cells converge on the TSC complex to regulate mTORC1.

In Chapter 2, I establish that isolated physiological activation of the Ras-ERK pathway stimulates mTORC1 signaling through spatial regulation of the TSC complex. I demonstrate that EGF stimulates the acute dissociation of the TSC complex from the lysosomal surface, where it interacts with Rheb. Through the use of multiple pathway inhibitors, I specifically establish that effector kinases of the Ras-ERK pathway regulate the TSC complex at the level of its lysosomal localization. Contrary to previously proposed models, I find that the regulation of the TSC complex by EGF does not involve even slight effects on TSC complex integrity, suggesting that
another mechanism must be in place to robustly activate mTORC1. I provide insights on the involvement of differential phosphorylation-dependent mechanisms in the spatial control of the TSC complex in propagating the growth factor signal to mTORC1. Lastly, I also identify a possible mechanism for direct PKC-mediated activation of mTORC1 through the spatial regulation of the TSC complex.

In Chapter 3, I discuss the implications surrounding the phospho-dependent spatial regulation of the TSC complex, including its relevance in advancing therapeutic insights. Recognizing that the signals that modify the function of the TSC complex largely reflect phosphorylation events, I discuss some phospho-proteomic data that suggest that there are additional residues on TSC2 that are responsive to growth factor signaling pathways and are likely to contribute to its regulation of Rheb and mTORC1. I finish by discussing the broader implications of these findings in the context of the mTORC1 signaling network.
1.4 References


CHAPTER 2:

ERK AND PKC SIGNALING ACTIVATE mTORC1 THROUGH SPATIAL REGULATION OF THE TSC COMPLEX

Experiments for Fig 2.5A & Fig 2.6C-E were done by Sophie Lockwood.

Gerta Hoxhaj provided technical assistance in obtaining samples in Fig 2.7C.
2.1 **Abstract**

The mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) serves as a molecular link between cellular growth conditions and anabolic processes that are fundamental to cell growth and proliferation. This evolutionarily conserved protein kinase complex sits at the convergence point of a vast signaling network that functions to integrate a wide array of signals to properly control cell, tissue, and organismal growth\(^1\)\(^2\). mTORC1 senses many upstream signals through a small G protein switch involving the tuberous sclerosis complex (TSC), composed of tumor suppressors TSC1 and TSC2, and TBC1D7, and the Ras-related small G protein Rheb, an essential activator of mTORC1. The TSC complex functions as a GTPase-activating protein (GAP) for Rheb, thereby converting Rheb to its GDP-bound state incapable of activating mTORC1. The TSC complex is acutely regulated by at least two of the major growth factor signaling pathways in cells, the PI3K-Akt and Ras-ERK pathways, through inhibitory phosphorylation events on TSC2. Specifically, the Akt-mediated phosphorylation of TSC2 dissociates the TSC complex from Rheb at the lysosome, allowing Rheb to become GTP-loaded and activate mTORC1\(^3\). The molecular mechanisms underlying the Ras-ERK pathway’s regulation of mTORC1, including the redundancy and hierarchy of these signaling events in the context of PI3K activation or pharmacological inhibition, remains poorly understood. Here, we find that, like PI3K-Akt signaling, isolated activation of the Ras-ERK pathway stimulates mTORC1 signaling through spatial regulation of the TSC complex. Our results indicate that the TSC complex is acutely regulated by Ras-ERK signaling at the level of its lysosomal localization. These findings point to the TSC complex and mTORC1 as a critical point of redundant regulation by the PI3K-Akt and Ras-ERK pathways, which may underlie both the physiological response to growth factors and their frequent oncogenic activation in cancer.
2.2 Introduction

Critically positioned at a key nexus between upstream growth inputs and downstream metabolic processes, the highly conserved mechanistic target of rapamycin complex 1 (mTORC1) is a central cell growth regulator. The activation of mTORC1 promotes the conversion of available nutrients into biomass through the stimulation of protein, lipid, and nucleotide synthesis, processes that are fundamental to cell growth and proliferation\(^4\)\(^-\)\(^10\). Given the great demand for nutrients and energy that accompanies the induction of these anabolic processes, the activation state of mTORC1 in normal cells is very tightly controlled.

There are two systems of small G proteins, the Rag and Rheb GTPases, that are key to the integrated control of mTORC1 by intracellular nutrients and exogenous growth factors. The coordinated actions of the Rag proteins, downstream of amino acids, and Rheb, downstream of growth factors, for regulation of mTORC1 on the surface of lysosomes have been reviewed in detail elsewhere\(^2\)\(^,\)\(^11\)\(^,\)\(^12\). In brief, the Rag GTPases are responsible for localization of mTORC1 to the lysosome when adequate levels of intracellular amino acids are present. While this is not sufficient to activate mTORC1, this translocation brings mTORC1 in proximity to Rheb, which in its GTP-bound state is a potent and essential activator of mTORC1\(^13\)\(^,\)\(^14\). Integration of the amino acid signal with growth factor signaling comes at the level of Rheb regulation at the lysosome. The TSC Complex (TSC1-TSC2-TBC1D7) acts as a GTPase-activating protein (GAP) for Rheb, thereby converting Rheb to its GDP-bound state incapable of activating mTORC1. The TSC complex is acutely regulated by at least two of the major growth factor signaling pathways in cells, the PI3K-Akt and Ras-ERK pathways, through inhibitory phosphorylation events on TSC2\(^15\)\(^-\)\(^19\). However, despite the establishment of the TSC complex and Rheb as the main players that control the activation mTORC1 by growth factors, the
mechanistic basis surrounding this regulation has only been recently defined. Specifically, previous work from our lab has shown that the major mechanism by which the PI3K-Akt pathway stimulates mTORC1 is through a phosphorylation-dependent release of the TSC complex from Rheb at the lysosome, thereby allowing Rheb to become GTP-loaded and turn on mTORC1 signaling\(^3\).

Since mTORC1 is a well-established downstream target of PI3K-Akt, hence frequently referred to as PI3K-mTOR, it is commonly thought to only be influenced by the PI3K-Akt pathway. However, there is significant evidence that the Ras-ERK pathway can also regulate mTORC1 activation. There have been previously identified phosphorylation sites for ERK and its downstream effector kinase RSK on TSC2, with some evidence that these sites contribute to the activation of mTORC1 by growth stimuli\(^{18,19}\). Additional studies have reported on ERK and RSK phosphorylation sites on the mTORC1 component Raptor, which also contribute to mTORC1 activation\(^{20,21}\). However, despite these studies, the molecular mechanisms underlying the Ras-ERK pathway’s regulation of mTORC1, including the redundancy and hierarchy of these signaling events in the context of the PI3K-Akt pathway, are poorly defined. Decoding the nature of signal rewiring within this network is particularly important in understanding the responses of tumors to clinical compounds that target both oncogenic pathways. Here, through endogenous localization of TSC2, we show that the TSC complex dissociates from lysosomal Rheb in response to growth stimuli specific to the Ras-ERK pathway, and that this dissociation acutely activates mTORC1 signaling. We show that Ras-ERK signaling regulates the TSC complex through phosphorylation-dependent spatial control, rather than TSC complex disassembly. Our data suggest that lysosomal release of the TSC complex could be a general mechanism for activation of mTORC1 by growth factor-regulated kinases. Fundamentally, our
data point to the TSC complex and mTORC1 as a key point of redundant regulation by the PI3K-Akt and Ras-ERK pathways, which could contribute to the development of therapeutic resistance when these pathways are targeted individually.

2.3 Materials and Methods

Cell Culture

All cell lines were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM; Corning/Cellgro, 10-017-CV), without pyruvate, with 10% fetal bovine serum (FBS; Sigma). Tsc2−/−(p53−/−) MEFs and their littermate-derived wild-type counterparts were kindly provided by D.J. Kwiatkowski of Harvard Medical School. For insulin, EGF, and PMA stimulation, subconfluent cells washed twice with PBS, serum starved in DMEM for 16 hr and without a media change were stimulated with either 1 µM insulin (Alpha Diagnostic, INSL-16-N-5), 100ng/mL EGF (Thermo Scientific, PHG0311) or 100ng/mL PMA (Sigma, P1585) for 15 min unless otherwise indicated. Inhibitors used in specific cell culture experiments include U0126 (Cell Signaling Technology) and, from Selleckchem, MK2206, Trametinib, G06983, Vemurafenib, PD183452, LJI308, SCH772984. Inhibitors were all resuspended in DMSO as 1000x stock solutions and store in -80°C.

Immunofluorescence Microscopy

HeLa cells cultured at 70% confluency were seeded into 6 well culture dishes containing glass coverslips (Zeiss, 474030-9000-000) at 0.1 million cells per well million cells per well and
allowed to attach and recover (16-14 hours). MEFs were seeded at 0.1 million cells per well.
Following attachment, cells were washed twice with PBS and incubated in serum-free medium
no longer than 16 hr prior to the treatments indicated. Cells were then washed 3 times with PBS
and fixed with 4% paraformaldehyde in PBS (ThermoScientific, 28908) for 15 min at room
temperature. Cells were then washed 3 times with PBS for 5 mins each and permeabilized with
PBS containing 0.2% Triton X-100 for 10 min at room temperature. Following 1 hr blocking in
Odyssey blocking buffer (Li-Cor, 927-40000) diluted 1:1 with PBS at room temperature,
coverslips were incubated for 16 hr at 4°C in primary antibodies diluted in blocking buffer
containing 0.2% Triton X-100. Following three 5 min washes in PBS, coverslips were incubated
for 1 hr at room temperature in the dark in secondary antibodies diluted in blocking buffer
containing 0.2% Triton X-100. Following 2 washes with PBS, coverslips were incubated with
DAPI (1:5000, Thermo Scientific D1306) for 1 min. Coverslips were washed again 2 more times
with PBS prior to mounting with Fluoromount G (SouthernBiotech, 0100-01).

The following primary antibodies were used for immunofluorescence (validation refers to
demonstration that immunofluorescent labeling in cultured cells is lost with knockout or RNAi-
mediated knockdown of the antigen): mouse anti-LAMP2 (H4B4 from Santa Cruz, sc-18822;
1:100; recognizes only human LAMP2), rat anti-LAMP1 (DSHB, 1D4B; 1:100; recognizes only
mouse LAMP1; validated in Albertti et al. 2010), rabbit anti-LAMP1 (CST, 9091; 1:200), rabbit
anti-TSC2 (CST, 4308; 1:1000; validated in Dibble et al. 2012\(^3\)), mouse anti-Rheb (Abnova,
H00006009-M01; 1:1000; validated in Menon et al. 2014\(^3\)), mouse anti-TBC1D7 (Sigma,
SAB1400543; 1:500; validated in Menon et al. 2014\(^3\)). The following secondary antibodies were
used for immunofluorescence: anti-rabbit, anti-mouse and anti-rat Alexa Fluor 488 (Life
Technologies/Molecular Probes, A21206, A11001, A11006; 1:1000), anti-mouse Alexa Fluor
594 (Life Technologies/Molecular Probes, A11032; 1:1000), and anti-rabbit Cy3 (Jackson ImmunoResearch, 111-165-144; 1:1000). All antibody stocks that were not in glycerol were divided into single-use aliquots and frozen. Once thawed for use these aliquots were used immediately and were not reused or refrozen.

Confocal images were acquired using a Nikon Eclipse Ti inverted microscope running NIS elements software, version 4.40. Z-stacks were captured at 0.25 µm intervals through a 60x/1.40 N.A. oil objective lens at 2048x2048 pixel resolution, with 100 nm pixel size and 12-bit depth, using a pinhole of 30 µm diameter and 4.1s dwell time. Andor Zyla 5.5 megapixel sCMOS camera was used. The raw .nd2 images were uploaded to Fiji/imageJ for quantification analysis. The center plane of a Z-stack containing at least 8-10 slices was used. For each image, regions of interest (ROI) were drawn around the cell border to accurately distinguish between cells and background noise. Each ROI was subjected to coloc2 analysis in Fiji/imageJ. The colocalization percentages were determined using Mander’s colocalization coefficient reported on coloc2 – for example, if determining TSC2 and LAMP2 colocalization, the number of pixels that overlap from the green channel (LAMP2) over the pixels from the red channel (TSC2) divided by the total number of pixels detected in the green channel above the background value x100. In each condition, a minimum of four different representative fields were captured and at least 50 cells were analyzed. Identical instrument settings and exposure times were used for all comparative analyses. All immunofluorescence experiments, have been repeated multiple times with representative results presented, and in merged color images, colocalization is indicated by yellow and orange regions.
DNA Constructs

All cDNA constructs are human sequences. The TSC2-S939A/S981A/S1130A/S1132A/T1462A (TSC2-5A) mutant was described previously (Zhang et al., 2009\textsuperscript{24}). Reconstituted isogenic lines of \textit{Tsc2\textsuperscript{-/-}}(\textit{p53\textsuperscript{-/-}}) MEFs were generated by infection with retroviruses encoding empty vector, wild-type TSC2 or TSC2-5A followed by selection with 100 to 250 \(\mu\)g/ml hygromycin B (Life Technologies/Invitrogen, 10687-010). Lower concentrations of hygromycin B (100 mg/ml) were used to select for cells expressing lower levels of TSC2, comparable to endogenous, to visualize and quantify changes in localization by immunofluorescence.

Cell Lysis, Fractionation, Immunoprecipitation, and Antibodies

For protein extracts lysates, cells were rinsed twice with cold PBS and lysed in ice-cold NP-40 lysis buffer (40 mM HEPES, pH 7.4, 120 mM NaCl, 1 mM EDTA, 1% NP-40 (Igepal CA-630), 5% glycerol, 10 mM sodium pyrophosphate, 10 mM glycerol 2-phosphate, 50 mM NaF, 1mM sodium orthovanadate, 1 \(\mu\)M Microcystin-LR (Enzo Life Sciences, ALX-350-012-C500) and 1:100 protease inhibitor cocktail [Sigma, P8340]), except for size-exclusion chromatography and GST affinity purification experiments in which cells were lysed in CHAPS lysis buffer (see below). Lysates were centrifuged at 20,000 g for 15 min at 4°C to remove insoluble material and protein concentrations were determined with Bradford assay (Biorad, 500-0006). Normalized protein lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and subjected to immunoblotting using the following primary antibodies: p-S6K1 T389 (CST, 9234), S6K1 (CST, 2708), 4EBP1 (CST, 9644), TSC2 (CST, 3612), p-TSC2 S664 (CST, 40729), p-TSC2 S1798 (Santa Cruz, sc-293149), p-TSC2 S939 (CST, 3615), p-TSC2 T1462 (CST, 3617) Rheb (Abnova, H00006009-M01), p-Akt S473 (CST, 4060), Akt (CST, 4691), ERK1/2 (CST,
9102), p-ERK T202/Y204 (CST, 9101), RSK (CST, 9355), p-RSK S380 (CST, 9335), YB-1 (Santa Cruz, sc-101198), p-YB1 S102 (CST, 2900) β-actin (Sigma, A5316). HRP-conjugated anti-mouse (CST, 7076) and anti-rabbit (CST, 7074) secondary antibodies were used.

For immunoprecipitation of endogenous TSC complex, cell lysates were incubated with either TSC1 antibody (R&D systems MAB4379) or TSC2 (R&D systems, MAB40401) antibody at 4°C rocking for 2-4 hr plus an additional 2 hr following addition of 25 µl of 50% slurry pre-washed protein A/G-agarose beads (Thermo Scientific/Pierce, 20421). Beads with immunocomplexes were centrifuged briefly at 3,000 g, washed three times in lysis buffer, and eluted in 2X laemmli SDS-sample buffer at 80°C for 10 min. In Figure 2.7B, cells were lysed in RIPA lysis buffer (150 mM NaCl, 10mM Tris pH 7.5, 1% NP-40, 1% deoxycholate, 0.1% SDS, 1x Roche protease cocktail inhibitor, Sigma phosphatase inhibitor cocktail #1).

Size-Exclusion Chromatography

Cells from a near-confluent 10-cm dish were washed with cold PBS and lysed on ice in 500 µl CHAPS lysis buffer (40 mM HEPES, pH 7.4, 120 mM NaCl, 1 mM EDTA, 0.3% w/v CHAPS, 10 mM pyrophosphate, 10 mM glycerol-2-phosphate, 50 mM NaF) and freshly added protease inhibitors at 1:100 (Sigma, P8340). Lysates were rocked at 4°C for 15 min and centrifuged at 20,000 g for 15 min to remove insoluble material. Supernatants were normalized for protein concentration between samples, and then passed through a 0.22 µM filter. Samples were processed and analyzed using a Fast Protein Liquid Chromatography (FPLC) system with a purifier and collection system connected to a 24-ml Superose 6 10/300 GL column (GE Healthcare, 45-002-901), which has a separation range from 5 kDa to 5 MDa. Before each run the system was equilibrated with three column volumes of lysis buffer. For each sample, 250 µl
(1% of column volume) was loaded into a 1 ml sample loop, and injected into the column with 1 ml of lysis buffer. Samples were eluted from the column and collected as 0.5 ml fractions using cold CHAPS lysis buffer with supplemented with Roche cocktail protease inhibitors. For SDS-PAGE, following addition of laemmli SDS sample buffer to the fractions and heating for 10 min at 80°C, all fractions from the same treatment were loaded on a single 26-lane 4-15% Criterion gel (Bio-Rad). To generate a standard curve, the elution volumes were determined for purified proteins and protein complexes of known molecular weights which were run in CHAPS lysis buffer unless otherwise noted. All standard samples contained at least 100 mM NaCl to minimize weak interactions with the column media and were passed through a 0.22 µM filter immediately prior to use. Peak position of these standard proteins was determined using connected FPLC software based on absorbance at 280 nm. In some cases the peak elution volume was confirmed by determining the protein concentrations of collected fractions with Bradford reagent. Elution volumes were transformed into Kav values (elution volume-void volume/column volume-void volume) and graphed against the base-10 logarithms of the predicted molecular weights (kDa). A second-order polynomial curve was fitted to the data with the equation from this curve used to estimate the corresponding molecular weights of fractions in Figure 2.7C.

**GST-Rheb Affinity Purifications**

HeLa cells were grown to near confluency, washed twice with PBS, and incubated in serum-free medium for 16 hr before indicated treatment. Cells were lysed in ice-cold CHAPS lysis buffer (40 mM HEPES, pH 7.4, 120 mM NaCl, 1 mM EDTA, 0.3% CHAPS, 5% glycerol, 10 mM sodium pyrophosphate, 10 mM glycerol 2-phosphate) with 1 µM Microcystin-LR (Enzo Life Sciences, ALX-350-012-C500) and 1:100 protease inhibitor cocktail (Sigma, P8340).
lysates were then centrifuged at 20,000 g for 15 min to remove insoluble material, and normalized for protein concentration between samples using Bradford assay. GST-Rab5A and GST-Rheb expressed in *E. coli* were purified as described in Dibble et al. 2012. GST-proteins were captured from bacterial lysates with glutathione-agarose beads, washed 3X in Rheb wash buffer (50 mM HEPES, pH7.4, 500 mM NaCl, 2 mM EDTA, 0.2% Triton X-100 with 1 mM DTT and 1:100 protease inhibitors added fresh), washed 2X in loading buffer (50 mM HEPES, pH 7.4, 1 mM EDTA, 5 mg/ml BSA, and 1:200 protease inhibitors), and loaded with 500 µM GDP in loading buffer for 5 min at 37°C with gentle agitation. Following loading, samples were placed on ice and nucleotide binding was locked in by adding 20 mM MgCl2 for at least 5 min before use. Loaded GST-Rheb or GST-Rab5a control on glutathione-beads (10 µl 1:1 bead/buffer slurry) were added to 1 ml of lysate (normalized to 2.5-3 mg/mL) to which either 5 mM EDTA had been added for the nucleotide-free treatment or 10 mM MgCl2 and 500 µM of the appropriate nucleotide for the nucleotide loaded treatments. Samples were rocked for 4 hr at 4°C. Beads were washed briefly 4 times with CHAPS lysis buffer plus 10 mM MgCl2 and then heated in 15 µl 1X SDS sample buffer at 70°C for 10 min.

2.4 **Results**

2.4.1 **EGF and PMA activate mTORC1 by inducing dissociation of the TSC complex from the lysosome**

To probe the molecular mechanism underlying the Ras-ERK pathway’s activation of mTORC1, we first established the signaling events surrounding mTORC1 activation in response
to different growth stimuli. While serum potently activates both the PI3K-Akt and Ras-Erk pathways, most individual growth factors and their downstream RTKs or GPCRs preferentially activate one or the other. Insulin has a well-established effect on activating mTORC1 through Akt-mediated phosphorylation of TSC2, while EGF is believed to activate mTORC1 through ERK and RSK in most settings. Tumor-promoting phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), also turn on the ERK pathway through direct activation of PKC. Indeed, after an acute stimulation (15 mins) of serum-starved HeLa cells, insulin strongly activated Akt, while EGF and PMA both preferentially activated ERK, with subsequent activation of mTORC1, as monitored by direct phosphorylation of its downstream substrate S6K1. To determine whether these acute signaling events alter the spatial distribution of the TSC complex, we localized endogenous TSC2 using a previously validated antibody for immunofluorescence. Consistent with our previous findings, under basal, serum-starved conditions, a significant population of TSC2 colocalizes with the lysosomal marker LAMP2. In response to insulin-mediated Akt activation, this localization is dramatically reduced, with subsequent activation of mTORC1 signaling. We next performed a time course of PMA (Fig. 2.1D-F) and EGF (Fig. 2.1G-I) stimulation and quantified TSC2 localization. With similar kinetics to insulin, the extensive TSC2-LAMP2 colocalization observed under serum-starved conditions is acutely reduced in response to both PMA and EGF, with a corresponding activation of mTORC1 signaling.

While the preferential activation of the Ras-ERK and PI3K-Akt pathways can be differentially controlled by the use of specific growth stimuli, we observed that stimulation with EGF, but not PMA, can transiently activate the Akt pathway at the earliest time points (Fig 2.1G). Thus, to determine whether isolated activation of the Ras-ERK pathway can indeed
stimulate TSC complex dissociation from the lysosome to activate mTORC1, independent of Akt signaling, we repeated the EGF stimulation in the presence of the Akt inhibitor MK2206\textsuperscript{30}. A time course of EGF stimulation following a brief pre-treatment of cells with MK2206 still activated mTORC1 signaling, albeit with somewhat delayed kinetics (Fig 2.1J). Importantly, this coincided with the ability of EGF to induce the dissociation of the TSC complex from the lysosome (Fig 2.1K, L). Taken together, these findings indicate that EGF and PMA stimulate dissociation of the TSC complex from the lysosome, coinciding with activation of mTORC1 in a manner that is independent of PI3K-Akt signaling.

We next sought to test the reproducibility of our findings in other cell lines and validated immunofluorescence systems. A time course of EGF stimulation in mouse embryonic fibroblasts (MEFs) (Fig 2.2A,B) and HEK 293Es (Fig 2.2C,D) revealed that the intense TSC2-LAMP2 colocalization under basal conditions is acutely reduced in response to EGF. Furthermore, similar to our findings in HeLa cells, this acute dissociation of TSC2 from the lysosomes coincides with activation of mTORC1 signaling. To test whether the entire TSC complex is under spatial influence of EGF and PMA, we performed previously described immunofluorescence experiments with a validated TBC1D7 antibody\textsuperscript{3}. Like TSC2, EGF and PMA stimulation acutely reduced the lysosomal localization of TBC1D7, in largely similar kinetics to insulin (Fig 2.2E). Collectively, these experiments further strengthen our findings, that EGF and PMA stimulation can spatially regulate the TSC complex in a manner that coincides with mTORC1 activation.
Figure 2.1 EGF and PMA acutely disrupt the lysosomal localization of TSC2 and stimulate mTORC1.

(A) Serum starved HeLa cells were acutely stimulated with insulin (100nM), EGF (100ng/mL) or PMA (100ng/mL) for 15 minutes, prior to measuring activation of mTORC1 signaling. (B) A timecourse of insulin stimulation (100nM) was done in serum starved HeLa cells and scored for activation of Akt and mTORC1. (C) Cells as in (B) were subjected to immunofluorescent labeling of endogenous TSC2 and LAMP2. Percent colocalization is graphed as mean ± SEM. Statistical significance was determined by using a two-tailed Student’s t test. *p < 1 x 10^{-7} compared to unstimulated, 0 min. Time courses of PMA (D, E, F), EGF (G, H, I), and EGF with MK2206 (2µM, 30 mins) pretreatment (J, K, L) were done in serum starved HeLa cells and measured for activation of mTORC1 and colocalization of TSC2 and LAMP2. Representative images (15 min timepoint) are shown.
Figure 2.1 (Continued).
Figure 2.2 EGF and PMA acutely disrupt the lysosomal localization of TSC2 and TBC1D7

(A) Serum-starved MEFs were stimulated with a time course of EGF (100ng/mL) and scored for activation of mTORC1. (B) Cells as in (A) were subjected to endogenous immunofluorescent labeling of TSC2 and LAMP1. Percent colocalization is graphed as mean ± SEM. Statistical significance was determined by using a two-tailed Student’s t test. *p < 1 x 10^{-6} compared to serum-starved condition. (C) Serum-starved HEK 293Es were stimulated with a time course of EGF (100ng/mL) and scored for activation of mTORC1. (D) Cells as in (C) were subjected to endogenous immunofluorescent labeling of TSC2 and LAMP2. Percent colocalization is graphed as mean ± SEM. Statistical significance was determined by using a two-tailed Student’s t test. *p < 1 x 10^{-7} compared to serum-starved condition. (E) Serum-starved HeLa cells were acutely stimulated with insulin (100nM), EGF (100ng/mL), and PMA (100ng/mL) for 15 mins prior to immunofluorescent labeling of endogenous TBC1D7 and LAMP1. Percent colocalization is graphed as mean ± SEM. Statistical significance was determined by using a two-tailed Student’s t test. *p < 1 x 10^{-6} compared to serum-starved condition.
Figure 2.2 (Continued).
2.4.2 The Ras-ERK pathway and PKC signaling stimulate release of the TSC complex from the lysosome and activate mTORC1

To further dissect the mechanism by which EGF and PMA stimulate the activation of mTORC1, we used a variety of small molecule inhibitors selective for components of upstream pathways (Fig 2.3A). We found that pre-treatment with the Akt inhibitor MK2206 prior to insulin stimulation completely blocked Akt stimulation and mTORC1 activation but had only minimal effect on EGF-stimulated mTORC1 signaling (Fig 2.3B). As expected, the MEK inhibitor trametinib had no effect on the insulin-mediated activation of mTORC1. However, trametinib also failed to block the EGF-induced activation of mTORC1, despite inhibiting Erk activation (Fig 2.3B). Interestingly, whereas 15 min stimulation of cells with EGF fails to activate Akt, EGF robustly activates Akt when MEK-ERK signaling is inhibited (Fig 2.3B). Akt activation in this setting accounts for the sustained stimulation of mTORC1 signaling, as co-treatment with MK2206 and trametinib inhibited EGF-stimulated activation of mTORC1. These findings highlight the cross-talk and redundancy between the Ras-ERK and PI3K-Akt pathways, which can negatively regulate each other’s activity such that pharmacologically blocking one pathway can effectively activate the other.

To further investigate the interplay between these pathways in the regulation of mTORC1 through the TSC complex, we quantified EGF-induced changes in TSC2 localization in the presence or absence of MEK inhibitors. As described above (Fig 2.1G-I), EGF stimulation induces a decrease in TSC2-LAMP2 colocalization and activates mTORC1. However, despite inhibiting ERK pathway activation, the MEK inhibitor U0126 failed to block both the EGF-induced dissociation of TSC2 from the lysosome (Fig 2.3C, D) and the subsequent activation of mTORC1 (Fig 2.3E). As above, under these conditions of ERK pathway inhibition, Akt
Figure 2.3. Differential effects of pathway-specific inhibitors reveal the complex rewiring of PI3K-Akt and Ras-ERK signaling.

(A) Schematic of the PI3K-Akt and Ras-ERK pathway regulation of the TSC complex and mTORC1, with the pathway inhibitors used in this dissertation. (B) Serum-starved HeLa cells pre-treated with MK2206 (2μM) and Trametinib (5μM) for 30 mins, prior to insulin (100nM) or EGF (100ng/mL) stimulation for 15 mins. (C) As in (B), cells were pre-treated with U0126 (5μM) for 30 mins prior to EGF stimulation and immunofluorescent labeling of endogenous TSC2 and LAMP2. Representative images are shown with percent colocalization graphed as mean ± SEM. Statistical significance was determined by using a two-tailed Student’s t test. *p < 1 x 10⁻⁵ compared to serum-starved (D) and immunoblot to score for mTORC1 activation (E). (F) Serum-starved cells were pre-treated with both U0126 (5μM) and MK2206 (2μM) for 30 mins prior to EGF stimulation and endogenous labeling of TSC2 and LAMP2. Representative images are shown. (G) Percent colocalization graphed as mean ± SEM. Statistical significance was determined by using a two-tailed Student’s t test. *p < 1 x 10⁻⁶ compared to EGF-stimulated.
Figure 2.3 (Continued).
signaling is responsive to EGF, providing a likely mechanism for these effects. In order to properly assess the individual contribution of the Ras-ERK pathway to the spatial control of the TSC complex in response to EGF, cells were stimulated in the presence of the Akt inhibitor. Consistent with basal Akt signaling influencing TSC complex localization in the absence of growth factors, MK2206 pretreatment alone promoted strong lysosomal localization of TSC2. This localization was significantly reduced by EGF stimulation with an accompanying increase in mTORC1 signaling, and MEK inhibition blocked this response (Fig 2.2F-H). Thus, the Ras-ERK pathway, like PI3K-Akt signaling, acutely stimulates release of the TSC complex from the lysosome in its control of mTORC1.

To further dissect the role of Ras-ERK signaling in regulating the TSC complex and mTORC1 activation, we pre-treated cells with other small molecule inhibitors of pathway components prior to stimulation with EGF or PMA. To avoid contributions from Akt activation, cells stimulated with EGF were also treated with MK2206. The MEK inhibitor Trametinib, ERK inhibitor SCH772984, and RSK inhibitor LJI308 all suppressed the EGF-induced dissociation of TSC2 from the lysosome and decreased mTORC1 signaling (Fig 2.4A, B, E). Thus, ERK activation of RSK plays a central role in regulating TSC complex localization and mTORC1 activation in response to EGF. PMA stimulates the PKC-mediated activation of Raf and is frequently used to activate ERK signaling independent of RTKs. PMA does not activate PI3K-Akt signaling. Consistent with the molecular action of PMA, the pan-PKC inhibitor Go6983 strongly inhibited the PMA-induced dissociation of TSC2 from the lysosome and activation of mTORC1(Fig 2.4C,D,F). Surprisingly, however, inhibitors of MEK, ERK, and RSK only partially inhibited mTORC1 signaling in response to PMA and failed to block the stimulated decrease in TSC2-LAMP2 colocalization. Collectively, these inhibitor studies reveal Akt-
independent mechanisms influencing TSC complex localization and mTORC1 that are mediated by ERK and PKC signaling.

During the course of this investigation, we discovered that the ability of the anti-TSC2 antibody validated for immunofluorescence (CST #4308) to recognize endogenous TSC2 on immunoblots was impaired when lysates were prepared from cells stimulated for 15 min with EGF or PMA, but not insulin (Fig 2.5A). However, all other TSC2 antibodies tested did not show this effect (CST #3612 shown in figure). This antibody-specific loss of signal was reversed for EGF with the MEK inhibitor trametinib and PMA with the PKC inhibitor Go6983, suggesting that a post-translational modification downstream of these pathways was masking the epitope for this antibody. According to Cell Signaling Technologies, the CST #4308 antibody was raised against the carboxy-terminus of TSC2. Thus, stimulated modifications to this region might underlie this epitope-masking effect. Indeed, previous studies identified a Ser residue at the very end of the carboxy-terminus of TSC2 as being phosphorylated in response to PMA stimulation\textsuperscript{19,31}. Unfortunately, outside of the CST #4308 antibody, no other TSC2 antibody that we have tried gives a specific signal in immunofluorescence experiments that is lost with TSC2 knockdown or knockout. To determine whether this effect detected on immunoblots might influence immunofluorescence measurements with the CST #4308 antibody, we measured total mean intensity across cells for the endogenous TSC2 signal in serum-starved cells compared to those stimulated with insulin, EGF, or PMA. Importantly, no significant differences in mean pixel intensity were detected between these conditions, thus validating the use of this antibody to monitor dynamic changes in TSC2 subcellular localization in immunofluorescence experiments.
Figure 2.4. ERK and PKC signaling both spatially regulate the TSC complex

(A, B) Serum starved HeLa cells were pretreated with MK2206 (2μM) for 30 mins, combined with different inhibitors Go 6983 (500nM), Trametinib (5μM), SCH772984 (1μM) and LJI308 (5μM), prior to EGF stimulation (100ng/mL) for 15 mins followed by colabeling of endogenous TSC2 and LAMP2. Representative cells are shown. (C,D). Cells as in (A,B) were pretreated with inhibitors Go 6983 (500nM), Trametinib (5μM), PD183452 (1μM), SCH772984 (1μM) and LJI308 (5μM), prior to PMA stimulation (100ng/mL) for 15 mins followed by colabeling of endogenous TSC2 and LAMP2. Representative cells are shown. (A,C) Phospho-YB-1 immunoblots are provided as a direct substrate of RSK. (E) Percent colocalization of (B) is graphed as mean ± SEM. Statistical significance was determined by using a two-tailed Student’s t test. *p < 1 x 10^{-5} compared to EGF+MK2206 condition. (F) Percent colocalization of (D) is graphed as mean ± SEM. Statistical significance was determined by using a two-tailed Student’s t test. *p < 1 x 10^{-7} compared to PMA-stimulated condition.
Figure 2.4 (Continued).
Figure 2.5. Validation of TSC2 antibody in immunoblots and immunofluorescence studies.

(A) Serum-starved HeLa cells were pre-treated with compounds shown and stimulated with insulin, EGF or PMA. Levels of total TSC2 from two different Cell Signaling Technology antibodies are shown. (B) Serum starved cells were stimulated with insulin, EGF, or PMA, prior to immunofluorescent labeling of TSC2. Mean pixel intensity was calculated by imageJ/FIJI.
2.4.3 A subpopulation of Rheb localizes to the lysosome and its physical interaction with the TSC complex is regulated by growth stimuli

The subcellular localization of endogenous Rheb has been characterized in previous studies, where a subpopulation was found to colocalize with lysosomal markers in a manner that was dependent on its C-terminal farnesylation\textsuperscript{3,32,33}. Consistent with these studies, we detect Rheb localization to lysosomes and find that it remains largely unchanged upon acute stimulation with insulin, EGF, or PMA (Fig 2.6A, B). This finding supports the notion that the population of Rheb that is lysosomal is not influenced by cellular growth conditions that regulate mTORC1. Importantly, previous studies from our lab demonstrated that the lysosomal localization of the TSC complex is dependent on Rheb binding at this location. Since Rheb is a critical activator of mTORC1 that is regulated by the dynamic localization of its inhibitory GAP, the TSC complex, we next determined whether distinct signals from growth factors could influence the Rheb-TSC complex interaction. A previously established Rheb-binding assay demonstrated that the TSC complex binds preferentially to the GDP-bound form of Rheb\textsuperscript{3}. Interestingly, we found that the binding of recombinant GDP-loaded Rheb to TSC2 was diminished when cells were stimulated with either insulin, EGF in the presence of MK2206, or PMA, and that binding was restored with inhibitors of Akt for insulin, MEK for EGF/MK2206, and PKC for PMA treatment (Fig 2.6C-E). Collectively, these findings reveal that Rheb contributes to the spatial control of the TSC complex, and importantly links the TSC complex-Rheb circuit to mTORC1 activation by growth stimuli through distinct upstream pathways.
Figure 2.6. A subpopulation of Rheb localizes to the lysosome where it interacts with the TSC complex and influences spatial control.

(A) HeLa cells were serum-starved for 16 hours prior and stimulated with insulin (100nM), EGF (100ng/mL) or PMA (100ng/mL) for 20 min prior to endogenous co-labeling of Rheb and LAMP1. Representative images shown (B) Percent colocalization of (A) is graphed as mean ± SEM. Endogenous TSC2 was pulled down from lysates of insulin (C), EGF (D), or PMA (E) stimulated HeLa cells (20 min), with recombinant purified GST-Rheb pre-loaded with GDP, compared to control pull-down with GDP-loaded GST-Rab5A. GST-fused bait proteins were detected with Ponceau S stain. Where indicated, prior to growth factor stimulation, cells were pre-treated with their specific pathway inhibitors: MK2206 for insulin (C), Trametinib and MK2206 for EGF (D), and Go6983 for PMA (E).
Figure 2.6 (Continued).
2.4.4 Signaling through EGF and PMA does not affect TSC complex stability.

A previous study concluded that signaling through the Ras-Erk pathway regulates the TSC complex through Erk-mediated phosphorylation of TSC2 at S664 and that this modification inhibits the complex by causing physical dissociation of the TSC complex components\textsuperscript{18}. To assess the effects of EGF and PMA stimulation on TSC complex integrity, we immunopurified endogenous TSC complexes from unstimulated, EGF-stimulated, or PMA-stimulated cells using a TSC1 antibody. Importantly, there was no change in association of TSC complex components upon EGF and PMA stimulation (15 and 30 mins) (Fig. 2.7A). Furthermore, TSC2 phosphorylated on residues directly targeted by ERK (S664)\textsuperscript{18} and RSK (S1798)\textsuperscript{19} was detected within these immunoprecipitated complexes following stimulation. Additional TSC1 immunoprecipitations from cells that were PMA-stimulated for up to 2 hours also showed no changes in complex association (data not shown). Similarly, immunoprecipitations using a TSC2 antibody failed to detect a change in complex stability in cells that were stimulated with EGF over a time course of 1 hour (Fig 2.7B). To further probe for effects of EGF signaling on TSC complex integrity, we used size-exclusion chromatography as a complementary approach. Our previous studies using sucrose density gradient fractionation showed that the loss of any of the three TSC complex components will result in an evident shift of the remaining subunits to lower mass\textsuperscript{23}. Consistent with previous studies, we find that TSC1 exists only within the large TSC complex, which has a peak mass of around 2 MDa, and that pools of TSC2 and TBC1D7 exist outside of this complex. Importantly, the fractionation pattern of TSC complex components was nearly identical between the serum starved and EGF-stimulated conditions, indicating that higher-order quaternary structure is unaffected by stimulation of Erk signaling. Thus, through multiple independent approaches, we establish that EGF and PMA signaling regulate the TSC
complex through effects on lysosomal localization and Rheb binding, rather than mechanisms involving complex disassembly or degradation.
Figure 2.7. EGF and PMA signaling do not affect TSC complex stability.

(A) HeLa cells were serum starved for 16h and stimulated with EGF (100ng/mL), PMA (100ng/mL) or insulin (100nM) for either 15m or 30m prior to lysis and immunoprecipitation with TSC1 antibody. (B) HeLa cells were serum starved for 16h and stimulated with a time course of EGF (100ng/mL) prior to lysis and immunoprecipitation with TSC2 antibody. (C) HeLa cells were serum starved for 16h and stimulated with EGF (100ng/mL) for 15m prior to lysis. Lysates were fractionated using size-exclusion chromatography. Estimated molecular weights were calculated from a standard curve.
Figure 2.7 (Continued).
2.4.5 PKC and ERK signaling induce dissociation of the TSC complex from the lysosome and mTORC1 activation independent of the five Akt phosphorylation sites on TSC2

The TSC complex relays signals from many upstream inputs to influence the GTP-loading state of Rheb to control mTORC1 activity. The signals that modify the function of the TSC complex generally result in specific phosphorylation events on TSC2. Some of the kinases responsible for these modifications on TSC2 and the residues that they modify have been previously identified and include Akt, ERK, RSK, and PKC (Fig 2.8A). Currently, phospho-specific antibodies are available to only four of these sites. In response to EGF, the rapid and direct, site-specific phosphorylation of TSC2 (Fig 2.8B) occurs in the same acute manner as dissociation of the TSC complex from the lysosome (Fig 2.4B). Accordingly, pre-treatment of cells with the MEK inhibitor Trametinib blocked EGF-stimulated phosphorylation of TSC2 on S664, while the PKC and RSK inhibitors had no effect (Fig 2.8B). EGF stimulation also led to TSC2 phosphorylation on the predicted RSK site, S1798, which was reversed by treatment with MEK-ERK-RSK pathway inhibitors. Similarly, PMA stimulation resulted in robust phosphorylation of the predicted ERK (S664) and RSK (S1798) sites, which was completely abolished when cells were pre-treated with the PKC inhibitor (Fig 2.8C). Interestingly, after both EGF and PMA stimulation, the canonical Akt sites (S939 and T1462) showed modest phosphorylation even in the presence of the Akt inhibitor, suggesting that other kinases can phosphorylate these canonical Akt sites, as suggested in previous studies for RSK\textsuperscript{19} and SGK3\textsuperscript{34} (Fig 2.8B). In this experiment, only the PKC inhibitor completely blocked phosphorylation of both sites, suggesting that either PKC directly and specifically targets these sites in response to these stimuli or that downstream kinases other than RSK are responsible. These findings
highlight the need to determine whether EGF and PMA are influencing TSC2 localization and mTORC1 activation through the same TSC2 sites phosphorylated by Akt in response to insulin.
Figure 2.8. ERK and PKC signaling induce dissociation of the TSC complex from the lysosomes through a phosphorylation-dependent mechanism.

(A) Schematic of some of the identified phosphorylation sites on TSC2. (B) Cell lysates from Fig 2.4A, serum-starved HeLa cells pre-treated with indicated pathway inhibitors for 30 mins prior to EGF stimulation. (C) Cell lysates from Fig 2.4C serum-starved HeLa cells pre-treated with indicated pathway inhibitors for 30 mins prior to PMA stimulation.
Our previous study on the insulin-mediated spatial control of the TSC complex found that phosphorylation of the five Akt-directed residues on TSC2 is required for the insulin-stimulated dissociation of TSC2 from the lysosome and subsequent activation of mTORC1. Consistent with these findings, Tsc2−/− MEFs expressing the TSC2-5A mutant lacking these sites display an attenuated activation of mTORC1 in response to insulin stimulation, relative to wild-type TSC2-expressing cells (Fig 2.9A). In contrast to these findings, EGF stimulated mTORC1 signaling to a nearly identical degree in the TSC2-WT and TSC2-5A expressing cells (Fig 2.9B). PMA similarly induced mTORC1 signaling at 15 min in the TSC2-WT and -5A reconstituted cells, but the TSC2-5A mutant cells displayed delayed activation at earlier time points (Fig 2.9C). Thus, despite stimulating modest phosphorylation of at least two of the canonical Akt sites (S939 and T1462; Fig 2.8B,C), EGF and PMA can acutely activate mTORC1 signaling independent of all established Akt sites on TSC2. Consistent with the complete suppression of mTORC1 signaling in the absence of growth factors, both TSC2-WT and TSC2-5A were found to associate with the lysosome when expressed in serum-deprived Tsc2−/− MEFs (Fig 2.9D). The TSC2-5A lysosomal localization was substantially stronger than TSC2-WT at baseline, indicating that phosphorylation of these residues might influence the basal-state localization of the TSC complex under growth factor withdrawal conditions, when it maximally inhibits Rheb and mTORC1. In these reconstituted Tsc2−/− MEFs, TSC2-WT behaved similarly to that observed for endogenous TSC2 in other cell systems, with insulin, EGF, and PMA all acutely stimulating a decrease in TSC2 lysosomal localization. As seen previously, the TSC2-5A mutant remained lysosomal in response to insulin. However, both EGF and PMA induced dissociation of the TSC2-5A mutant from the lysosome (Fig 2.9D), consistent with the sustained ability of these growth stimuli to activate mTORC1 signaling in these cells. Collectively, these studies reveal
additional molecular mechanisms underlying the PKC and ERK-mediated regulation of mTORC1, which also involve spatial control of the TSC complex.
Figure 2.9 EGF and PMA stimulation promote mTORC1 activation independent of the five Akt sites on TSC2.

(A) *Tsc2*<sup>−/−</sup> MEFs reconstituted with either wildtype TSC2 (WT) or mutant TSC2-5A were serum starved for 16 hours before stimulation with a time course of insulin (100nM). (B) *Tsc2*<sup>−/−</sup> MEFs reconstituted with either WT TSC2 or 5A were serum starved for 16 hours before stimulation with EGF (150 ng/mL) for the times shown. (C) *Tsc2*<sup>−/−</sup> MEFs reconstituted with either WT TSC2 or 5A were serum starved for 16 hours before stimulation with PMA (100 ng/mL) for the times shown. (D) TSC2-WT and TSC2-5A reconstituted *Tsc2*<sup>−/−</sup> MEFs were stimulated with insulin, EGF, or PMA for 15 mins prior to immunofluorescent labeling of TSC2 and Lamp1. Percent colocalization is graphed as mean ± SEM. * denotes p < 1 x 10<sup>−9</sup> as compared to insulin stimulated TSC2-WT expressing cells.
Figure 2.9 (Continued).
2.6 Summary & Conclusion

The TSC complex has emerged as a central hub that receives a variety of signals reflecting growth conditions, and in turn, relays these signals to properly regulate Rheb and mTORC1. Two of the major upstream growth factor signaling pathways, PI3K-Akt and Ras-Erk, regulate the TSC complex through phosphorylation of specific residues on TSC2, thereby disrupting the complex’s ability to act as a GAP for Rheb. This regulation appears to occur through mechanisms that do not directly influence the biochemical GAP activity of TSC2, per se, but rather the spatial colocalization of the TSC complex with Rheb. In a previous study, the Manning laboratory established that the PI3K-Akt pathway is regulated by insulin through spatial control of the TSC complex\(^3\). In this dissertation, we found that two other growth promoting pathways, Ras-ERK and PKC, similarly spatially regulate the TSC complex and activate mTORC1.

In contrast to a previously suggested model\(^{18}\), we demonstrate through a variety of approaches that EGF and PMA stimulation have no effect on TSC complex stability within the time frame in which mTORC1 is fully activated by these growth signals. While it is possible that chronic stimulation of growth factors and certainly longer term exposure to phorbol esters may eventually destabilize the TSC complex, we detect no such effects in time courses of up to 2 hours of stimulation (data not shown). Furthermore, a disassembly mechanism after chronic stimulation does not support the acute and rapid ERK- and PKC-mediated activation of mTORC1 that we observe.

More recent studies have further examined the contribution of ERK and PKC signaling in mTORC1 activation in multiple settings. In myoblasts, TSC2 phosphorylation on a previously mapped ERK site (Ser 664) was found to underlie the PKC and ERK-dependent activation of
mTORC1\textsuperscript{35}. While a mechanism was not explored, the stimulation and kinetics of mTORC1 activation that observed in myoblasts coincides with our own findings of the rapid dissociation of TSC2 from lysosomal Rheb upon PMA stimulation. Another study has reported on PKC-mediated mTORC1 activation that is dependent on phosphorylation of the canonical Akt-directed site (Ser 939) on TSC2\textsuperscript{36}. Our data indicate that there are additional mechanisms underlying the PKC- and ERK-mediated regulation of mTORC1 that are independent of the Akt sites but also still influence the lysosomal localization of the TSC complex.

As a heavily phosphorylated protein, it is not surprising that the hierarchy of signals and phosphorylation events on TSC2 is still an active area of investigation. Indeed, for the majority of mapped sites on TSC2, neither the kinase nor the molecular consequences surrounding phosphorylation are known. In light of our findings that the TSC complex can be acutely regulated by multiple growth signaling pathways through spatial control, a deeper understanding of the complexity of events surrounding this regulation is required. In the last chapter, I further discuss molecular insights on the convergence of growth signals on the TSC complex, including its relevance to studying resistance to targeted therapeutics.
2.7 References


CHAPTER 3:

DISCUSSION AND FUTURE DIRECTIONS
3.1 Overview

In this dissertation, I investigated the regulation of the TSC complex by ERK and PKC signaling (Fig 3.1). First, in multiple settings, I determined that in response to growth-promoting signals that activate the ERK and PKC pathways, the TSC complex acutely dissociates from the lysosome, which corresponds to a rapid and robust activation of mTORC1 signaling. I also showed that when ERK and PKC signaling branches are activated, there is reduced interaction between lysosomal Rheb and its direct regulator, the TSC complex, which occurs independent of PI3K-Akt signaling. While more experiments are required to map potential phosphorylation-dependent regulation of TSC2 downstream of these signaling pathways, data thus far point to a general mechanism by which upstream growth factor-regulated kinases activate mTORC1 through the stimulated release of the TSC complex from Rheb at the lysosome, where Rheb-GTP activates mTORC1. Specifically, a phosphorylation-based mechanism likely influences the lysosomal shuttling of the TSC complex in response to ERK and PKC signaling, and the accompanying rapid mTORC1 activation. In this last chapter, I provide further insights into the molecular consequences surrounding multi-site phosphorylation of TSC2 in the context of spatial regulation of the TSC complex. Furthermore, I discuss how this spatial control mechanism can advance our understanding of resistance to targeted therapeutics. Lastly, I finish by discussing relevant future directions of research in the TSC-Rheb-mTORC1 circuit.
Figure 3.1 Graphical summary of the dissertation.
3.2 Growth signal convergence on the TSC complex: molecular consequences and therapeutic insights

3.2.1 Multi-site phosphorylation events dynamically regulate the TSC complex

In normal physiological states, mTORC1 signaling is tightly regulated by dynamic changes in cellular growth conditions\(^1\). Studies over the past 15 years have demonstrated that the integrated control of mTORC1 involves two sets of small G proteins that interact directly with the kinase complex. The Rag GTPases recruit mTORC1 to the lysosomal surface when sufficient amino acid levels are present\(^2,3\), and it is here where an additional signal in the form of direct interaction with the Rheb GTPase is required to fully activate mTORC1. Thus, once at the lysosomes, the activation state of mTORC1 is mostly dictated by the GDP or GTP-bound state of Rheb. To date, the only well-established regulator of Rheb is the TSC complex, which stimulates the intrinsically weak GTPase activity of Rheb to convert it from its GTP-bound active state to its GDP-bound inactive state\(^4-9\). Through its inhibition of Rheb, the TSC complex is a critical upstream negative regulator of mTORC1 and loss of TSC complex components leads to growth factor-independent activation of mTORC1\(^10,11\).

The TSC complex has emerged as a highly integrated signaling node that receives a variety of intracellular and extracellular signals to influence the GTP-loading state of Rheb and the activation state of mTORC1\(^12\). The signals that modify the function of the TSC complex largely reflect phosphorylation events, with nearly 40 distinct sites mapped thus far on TSC2 alone (www.phosphosite.org). While some of the kinases responsible for phosphorylating these sites have been identified (i.e., Akt, AMPK, ERK, GSK3, CDK1, PKC, RSK, etc.), for the majority of these sites, neither the kinase nor the molecular significance of phosphorylation are known\(^12\).
In a previous study, the Manning laboratory found that localization of the TSC complex to the lysosomal surface is necessary and sufficient for its inhibition of mTORC1 signaling, and its release from the lysosome is essential for the PI3K-Akt pathway to activate mTORC1. The events surrounding this regulation involve direct phosphorylation of TSC2 by Akt on five Ser/Thr residues. Through the work in this dissertation, we have found that other growth signaling pathways also regulate the TSC complex through spatial control. While our results thus far suggest that an acute phosphorylation-dependent mechanism might underlie this regulation, we observe that EGF and PMA signaling still promote dissociation of TSC2 from lysosomal Rheb and mTORC1 activation even when Akt is pharmacologically inhibited and the five canonical Akt sites are mutated. Therefore, while the Akt sites could still be involved in the ERK and PKC-mediated spatial control of the TSC complex, our data suggest that these sites alone are not sufficient to propagate the EGF and PMA signal and activate mTORC1.

As part of this study and to gain a more complete picture of the hierarchy of events surrounding the regulation of the TSC complex by growth stimuli, we performed LC-MS/MS phosphorylation profiling of TSC2 from HEK 293E cells that overexpressed Flag-TSC2 and acutely stimulated (15 mins) with EGF or PMA. Following immunopurification, we were only able to obtain less than 70% peptide coverage, which made some previously characterized sites (i.e. Ser 540, Ser 1798) undetectable. Nevertheless, in this profiling, we identified 14 phosphopeptides of TSC2 that were dynamically regulated in response to EGF and PMA stimulation relative to unstimulated cells (Table 3.1). Of note is the immense degree of overlap between the peptides that were phosphorylated in response to EGF or PMA. Importantly, as a positive control, we were able to detect previously characterized ERK (Ser 664) and
Table 3.1 List of identified phosphopeptides after EGF and PMA stimulation.

Flag-TSC2 was overexpressed in HEK 293E cells. After serum-starvation and acute stimulation with EGF or PMA (15 mins), FLAG was immunopurified, subjected to SDS-PAGE, gel excision and trypsin digest prior to LC-MS/MS. Total peptide coverage among all conditions ranged from 59-63%. The phosphopeptides identified above were found enriched in the stimulated condition relative to serum starved. In the 3rd and 4th columns, the number of phosphopeptides is provided for either stimulus, with the top number corresponding to stimulated conditions, and the bottom number corresponding to serum starved conditions. Green corresponds to sites that were identified as being phosphorylated and enriched with the stimulus, yellow corresponds to not. TSC2 sites with evidence of in vivo phosphorylation by known kinases are also indicated. Human numbering is used for reference.

<table>
<thead>
<tr>
<th>Phosphopeptides</th>
<th>TSC2 Site</th>
<th>EGF</th>
<th>PMA</th>
<th>Kinase</th>
<th>Reference(s)</th>
</tr>
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<tbody>
<tr>
<td>KKTSGPLsPPTGPG</td>
<td>Ser 664</td>
<td>9/3</td>
<td>6/3</td>
<td>ERK</td>
<td>Ma et al, 2005</td>
</tr>
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<td>SFRARSTsLNERPKS</td>
<td>Ser 939</td>
<td>2/1</td>
<td>4/1</td>
<td>Akt/RSK</td>
<td>Manning et al, 2002; Inoki et al, 2002; Roux et al, 2004</td>
</tr>
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<td>PKQGLNNSPPVKEF</td>
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<td>6/2</td>
<td>5/2</td>
<td>?</td>
<td></td>
</tr>
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<td>Ser 981</td>
<td>2/1</td>
<td>4/1</td>
<td>Akt</td>
<td>Dan et al, 2002</td>
</tr>
<tr>
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<td>Ser 1130</td>
<td>2/1</td>
<td>6/1</td>
<td>Akt</td>
<td>Inoki et al, 2002; Dan et al, 2002</td>
</tr>
<tr>
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<td>3/2</td>
<td>5/2</td>
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</tr>
<tr>
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<td>2/1</td>
<td></td>
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<td>3/2</td>
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<td>3/2</td>
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<td>LPSSPSsPSGLRPR</td>
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<td>Manning et al, 2002; Inoki et al, 2002; Roux et al, 2004</td>
</tr>
</tbody>
</table>
and PKC (Ser 1364)\textsuperscript{14} sites as being phosphorylated in response to EGF and PMA respectively. In subsequent experiments, these modifications were lost when cells were pre-treated with a MEK inhibitor or PKC inhibitor before stimulation (Fig 3.2A, B).

Out of the 14 phosphopeptides we identified in this profiling, 13 of them have either been characterized extensively, with kinases responsible identified (Table 3.1), or reported in a prior phosphoproteomic analysis of TSC1 and TSC2 from PMA stimulated cells\textsuperscript{14}. Remarkably, since the first reporting of six of these sites in 2005, the regulatory inputs and kinases responsible for these phosphorylation events are still unknown. While some of these sites are likely to represent novel mechanisms or be important in already established mechanisms, it’s just as likely that some of these are purely unregulated sites that are involved in protein folding.

One out of the 14 identified phosphopeptides in our profiling was not reported in the original 2005 study\textsuperscript{14}, but has since been identified in more recent phosphoproteomic and mechanistic approaches\textsuperscript{15,16}. We found Ser 1452 to be phosphorylated after acute EGF and PMA stimulation, and this modification is blocked by pre-treatment with a MEK inhibitor and a PKC inhibitor respectively (Fig 3.3A). While the functional significance of this site, including the specific kinase responsible for its phosphorylation has not been identified, given our data and the sequence context, ERK is a candidate kinase for this phosphorylation event. The Ser 1452 phosphopeptide sequence (Table 3.1) fits the consensus sequence for ERK, P-X-S/T-P, where P at the +1 position is favored, with secondary preference for P at the -2 position\textsuperscript{17}. Interestingly, Ser 1452 was identified in a recent study as one of multiple TSC2 sites that play a critical role in eccentric contraction (EC)-induced activation of mTOR signaling in skeletal muscle\textsuperscript{16}. Of particular interest is the finding that similar to growth factors, EC can also cause rapid TSC2
Figure 3.2 Effects of EGF, U0126, PMA, and Go6983 on previously characterized ERK and PKC sites on TSC2.

FLAG-TSC2 was immunopurified from serum-starved (16 hours) human embryonic kidney (HEK)-293E cells, pre-treated for 30 mins with U0126 (5µM) or Go6983 (1µM), before stimulation with EGF (100ng/mL) or PMA (100ng/mL) for 15 mins. The ratios of phosphorylated to total peptide levels, measured as total ion current (TIC) by means of LC-MS/MS, of the indicated sites (A) Ser 664, (B) Ser 1364 on TSC2, under the different conditions are graphed. ND indicates phosphopeptide not detected.
Figure 3.2 (Continued).
Figure 3.3 Effects of EGF, U0126, PMA, and Go6983 on TSC2 phosphorylation sites.

FLAG-TSC2 was immunopurified from serum-starved (16 hours) human embryonic kidney (HEK)-293E cells, pre-treated for 30 mins with U0126 (5µM) or Go6983 (1µM), before stimulation with EGF (100ng/mL) or PMA (100ng/mL) for 15 mins. The ratios of phosphorylated to total peptide levels, measured as total ion current (TIC) by means of LC-MS/MS, of the indicated sites (A) Ser 1452, (B) Ser 1130 on TSC2, under the different conditions are graphed. ND indicates phosphopeptide not detected.
Figure 3.3 (Continued).

A

Ser 1452
Ratio of Phospho-Peptide Signal

B

Ser 1130
Ratio of Phospho-Peptide Signal

EGF: - + + - -
U0126: - - + - -
PMA: - - - + +
G06983: - - - - +
dissociation from late endosomal/lysosomal structures, through a phosphorylation-dependent mechanism\textsuperscript{18,19}. Whether Ser 1452 turns out to be a \textit{bona fide} ERK target or not, these findings collectively emphasize the expanding role for multi-site phosphorylation of TSC2 in spatial regulation of the TSC complex.

On theme with the convergence of the PI3K-Akt and Ras-Erk pathways on the TSC complex, multiple kinases have been found to converge and phosphorylate the same sites on TSC2. For instance, Ser 939 has been previously found to be phosphorylated by Akt and RSK (Table 3.1), and experiments from this dissertation (Fig 2.8C) suggest that PKC might also phosphorylate this site. These protein kinases have overlapping substrates because they are members of the protein kinase A, protein kinase G, protein kinase C (AGC) kinase family. The AGC kinase family consists of more than 60 protein kinases that are characterized by a conserved catalytic domain and, for most family members, activation by phosphorylation of two highly conserved regulatory motifs: an activation segment in the catalytic domain targeted by PDK1, and a hydrophobic motif\textsuperscript{20}. Once activated, these kinases in turn have a propensity to phosphorylate basophilic sites containing positively charged Arg or Lys residues in specific positions relative to the phospho-acceptor Ser/Thr. Many of the growth factor-regulated members of this family, including Akt, RSK, SGK, and S6K, phosphorylate a consensus motif of R/KXR/KXXS/T, and PKC isoforms can also phosphorylate specific substrates with this motif\textsuperscript{21}. There are many examples of specific sites on substrates being phosphorylated by more than one family member – for example, both S6K and RSK can phosphorylate the ribosomal S6 protein\textsuperscript{22} and Akt, RSK, PKC, and S6K can all phosphorylate the same Ser residue of GSK3 in different contexts\textsuperscript{23,24}. This profound ability of different AGC kinases to phosphorylate the same substrates has most likely evolved to allow for different extracellular signals to control the same
cellular machinery and elicit similar responses. This is clearly represented in the case of TSC2 as a downstream substrate of multiple AGC kinases. For example, the observed phosphorylation event on the Ser 939 residue of TSC2 by Akt\textsuperscript{25,26}, RSK\textsuperscript{27}, and PKC\textsuperscript{28} function to inhibit the TSC complex and consequently activate mTORC1 signaling in response to diverse upstream growth signals.

Interestingly, our profiling experiment has identified Ser 1130, a previously characterized Akt site (Table 3.1) as robustly phosphorylated after acute PMA stimulation. This phosphorylation is blocked by pre-treatment with a PKC inhibitor, suggesting that this site, aside from being phosphorylated by Akt in response to insulin is also regulated by PKC activity (Fig 3.3B). While these studies demonstrate the complexity of phosphorylation signals that converge on TSC2, it also highlights an attractive mechanism that enable cells and organisms to quickly respond to changes in cellular growth conditions. For example, as discussed in the previous chapter, the EGF- and PMA-mediated rapid phosphorylation of TSC2, resulting in immediate translocation of the TSC complex from lysosomal Rheb, coincides with mTORC1 activation. As such, we are only beginning to scratch the surface in mapping out the hierarchy of TSC2 phosphorylation events and their exact stoichiometrical contributions in propagating the signal downstream. From our findings with insulin\textsuperscript{4} and studies in this dissertation (i.e. Fig 2.9), what is apparent is that, the activation of mTORC1 from spatial regulation of the TSC complex is dependent on a combination of multiple phosphorylation events. A deeper understanding of this regulation, including any redundancies, is required especially given the emerging role of sustained mTORC1 signaling in the development of resistance to targeted therapeutics.
3.2.2 Redundancies in the upstream signaling network can promote sustained mTORC1 signaling and resistance to targeted therapeutics

While genetic disruption of the TSC complex underlies aberrant mTORC1 activation and tumor growth in the TSC disease\(^29\) and in lymphangioleiomyomatosis (LAM)\(^30\), a related lung disorder, in most human cancers the TSC complex is intact but is functionally inactivated due to genetic alterations influencing the upstream growth factor signaling pathways\(^12,31\). The PI3K-Akt and Ras-Erk pathways, which become constitutively activated through gain of function oncogenic mutations (e.g. PI3K, Akt, Ras, Raf) or loss of inhibitory tumor suppressors (e.g. PTEN, NF1), are believed to account for much of the uncontrolled activation of mTORC1 in cancer cells\(^31,32\). Since the TSC-Rheb-mTORC1 circuit is a major shared downstream target of two of the most commonly activated oncogenic signaling pathways, there has been immense interest in the use of mTORC1 inhibitors to treat cancer\(^32\). However, inhibition of mTORC1 alone is not sufficient to cause tumor regression. The many feedback loops, crosstalk mechanisms, and downstream convergence points of the PI3K-Akt and Ras-Erk pathways result in decreased efficacy of single-agent therapies targeting these pathways in disease. Because these upstream oncogenic pathways have many downstream targets, the feedback loops and crosstalk mechanisms that arise contribute to restored signaling through these, or other pathways, to mTORC1, and ultimately to tumor cell survival.

Interestingly, what has emerged from studies in multiple distinct cancer lineages is that, while mTORC1 inhibition may not be sufficient, it is likely necessary to achieve a clinical response to drugs that target the upstream oncogene\(^31\). The molecular details from these studies are discussed in chapter 1 of this dissertation. In brief, treatment with the mTORC1 inhibitor everolimus restored sensitivity to PI3K inhibitor BYL719 in resistant \(PIK3CA\) mutant breast
cancer lines and xenograft tumors\textsuperscript{33}. Later studies using the same inhibitor in squamous cell carcinomas of the head and neck bearing the same \textit{PIK3CA} mutations found similar results, with sustained mTORC1 signaling characterizing resistance\textsuperscript{34}. Recent studies on \textit{PIK3CA} mutant cell lines further demonstrated that active mTORC1 signaling as mediated by SGK1 underlies resistance of these cell lines to PI3K inhibitors\textsuperscript{35}. Similar findings were observed in melanoma cell models – those that were sensitive to BRAF inhibitors displayed diminished mTORC1 signaling post-treatment, while resistant cell lines were all characterized by sustained mTORC1 signaling\textsuperscript{36}. Collectively, these studies demonstrate that the status of mTORC1 activation may serve as a useful biomarker for resistance to targeted therapeutics.

Since personalized medicine approaches based on tumor genotype has increasingly become the standard of care, defining the molecular nature of resistance is critical to both predicting and improving clinical responses to cancer therapeutics. Resistance involving sustained mTORC1 activation is likely driven by the redundancy in the upstream signaling network. The reactivation of RTKs upstream of both the PI3K-Akt and Ras-ERK pathway upon inhibition of single nodes in either pathway is one example of said redundancy\textsuperscript{21}. Another way that can render mTORC1 signaling unresponsive to inhibition of the major oncogenic pathway is through bypass activation of parallel pathways (e.g. PI3K or Ras). Furthermore, cell culture studies in breast cancer, lung cancer, and melanoma also found that loss of function of the TSC complex contributed to resistance to targeted therapeutics\textsuperscript{33,36,37}. As discussed in chapter 1, a large number of oncogenes and tumor suppressors influence the activation state of mTORC1 through the TSC complex. Thus, any aberrant inhibition of the TSC complex (e.g. mutations in upstream network) will frequently underlie sustained mTORC1 signaling, and consequently therapeutic resistance.
The spatial regulation of the TSC complex that was characterized in our earlier studies and in this dissertation can be a useful tool to test the sensitivity or resistance of cell lines to targeted therapeutics. Specifically, in therapy resistant settings, one might expect to observe sustained dissociation of the TSC complex from lysosomal Rheb in the presence of the given therapeutic. Alternatively, if mTORC1 signaling is sensitive to specific pathway inhibitors, then these compounds will induce a rapid translocation of the TSC complex to the lysosome to put a brake on Rheb and shut down its activation of mTORC1. In Menon et al using PTEN-deficient prostate cancer cells, an example of such a response was shown. PTEN null cells exhibit constitutive PI3K-Akt signaling, leading to phosphorylation of TSC2 and its growth factor-independent dissociation from the lysosome (Fig 3.4A). The sustained dissociation of TSC2 even under serum starvation conditions was rapidly reversed by treatment with an Akt inhibitor, further demonstrating the highly reversible nature of this regulation.

I further tested this model using SkMel28 cells, a B-RAFV600E mutant metastatic melanoma line that has constitutive activation of the ERK pathway and mTORC1. Under serum starved conditions, these cells displayed chronic dissociation of TSC2 from the lysosome with accompanying high mTORC1 signaling (Fig 3.4B). Treatment with a specific RAF inhibitor, vemurafenib, led to rapid translocation of TSC2 to the lysosome and dampened mTORC1 activation (Fig 3.4B). These experiments, in two different cancer settings driven by different oncogenes, highlight the potential utility of the TSC complex spatial control mechanism as a tool for predicting sensitivity or resistance of cell models to a therapeutic. The results from these and other studies described in chapter 1 advance an approach to cancer therapy that not only focuses on the genetic diagnosis of tumors to choose a targeted therapeutic, but also of early monitoring of biomarkers that are predictive of therapeutic response. Furthermore, in addition to
physiological stimulation of the Ras-ERK pathway as demonstrated in experiments in chapter 2, mutations leading to growth factor-independent activation of the Ras-ERK pathway in cancer also induce mTORC1 signaling through dissociation of the TSC complex from the lysosome.
Figure 3.4 PTEN null cells and B-Raf mutant melanoma cells exhibit growth factor-independent dissociation of TSC2 from the lysosome.

(A) Figure from Menon et al, 2014. Serum starved PC3 cells were treated with (30 min) with vehicle (DMSO) or MK2206 (2μM) prior to colabeling of TSC2 and LAMP2. (B) Serum starved SkMel28 cells were treated (45 min) with vehicle (DMSO) or vemurafenib (5μM) prior to colabeling of TSC2 and LAMP2.
3.3 Future Directions

Much of the interest in the PI3K-Akt and Ras-ERK pathways upstream of mTORC1 has focused on their critical role in driving cancer cell growth and proliferation. More recently, this has extended to defining the molecular events and oncogenic lesions that occur in the upstream signaling pathways and how they contribute to resistance to targeted therapeutics. The work in this dissertation has established that the spatial regulation of the TSC complex is a general mechanism for growth factor-regulated protein kinases to activate mTORC1. Importantly, that this mechanism also applies to oncogenic activation of Raf helps advance our understanding of the molecular inputs that predict sensitivity or resistance of cancer cells to pharmacological agents.

Despite the progress made in defining the molecular basis of mTORC1 activation by upstream growth factor pathways, fundamental questions that are key to our understanding of the dynamic TSC-Rheb-mTORC1 circuit remain unanswered. Perhaps one of the most essential questions revolve on whether there are other regulators of Rheb aside from the TSC complex. As discussed in chapter 1, the signals that influence the activation state of mTORC1 converge on the regulators that control the GDP/GTP-status of Rheb and the Rag proteins. In contrast to the Rag GTPases, to date, neither true GEFs nor GDIs have been identified for Rheb. Identifying potential Rheb regulators will further expand our understanding of the essential molecular inputs that influence mTORC1 activation.

While we have established that the extensive TSC2-LAMP2 colocalization under basal serum-starved conditions keeps Rheb in its GDP-bound form thereby rendering mTORC1 inactive, we haven’t fully explored what all the molecular determinants are for this localization. Likewise, it will also be interesting to track what happens to the TSC complex after it dissociates
from the lysosome in response to growth stimuli. Whether it localizes to another compartment or remains cytosolically diffuse, or whether it translocates to a specific location depending on the intensity and timing of stimulation, are basic questions that could further inform our understanding of growth factor-mediated spatial regulation.

Another important clinical setting where spatial regulation of the TSC complex may be particularly important is in the TSC disease itself. The TSC disease is characterized by loss of function mutations in TSC1 or TSC2, with many missense mutations mapped on TSC2\textsuperscript{43-46}. While a lot of these mutations are in the TSC1 binding and GAP domains of TSC2, some are also found in the large, heavily phosphorylated region of the protein, which we refer to as the phosphoregulatory domain. Thus far, the molecular mechanism surrounding their loss of function is unknown\textsuperscript{47,48}. Similar to what we observe in oncogenic activation, it would be interesting to examine whether patient-derived mutations also cause chronic dissociation of the TSC complex from the lysosome, thus rendering them inactive for their regulation of Rheb and mTORC1.

This dissertation has helped establish that multiple growth factor signaling pathways influence mTORC1 activation through spatial control of the TSC complex, and has improved our understanding of how diverse upstream molecular inputs can affect not only the acute physiological activation of mTORC1 but also its oncogenic activation. We hope that this knowledge, together with the future research directions discussed herein, will lead to further advancement in cancer therapeutics.
3.4 References


