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The Role of MAP4K3 in mTORC1 activation

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by

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This Thesis of Elian Xuanyu Lee is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chapter 2 is an original document describing scientific work that is currently being prepared as a manuscript for submission in a much-revised form. Hsu, C.L., Ohnishi, K., Lee, E.X., Meisenhelder, J., Paz, E.P., Hunter, T., and La Spada, A.R.

"MAP4K3 regulates mTORC1 activity via AMPK signaling." The dissertation author is the co-author of this work.

ABSTRACT OF THE THESIS

The Role of MAP4K3 in mTORC1 Activation

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Elian Xuanyu Lee

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Professor Albert R. La Spada, Chair

Professor Yunde Zhao, Co-Chair

The ability of cells to maintain metabolic homeostasis in response to changes in nutrient availability is critical for cell survival. The mammalian target of rapamycin complex 1 (mTORC1) integrates various environmental stimuli to regulate cellular processes, including autophagy, cell growth, protein synthesis, and lipid metabolism. Of the various inputs to mTORC1, the amino acid sensing pathway is among the most

potent. This thesis describes three studies that aim to elucidate the molecular mechanisms by which cells regulate metabolic homeostasis in response to amino acids.

In Chapter 1, we give a brief overview on the current knowledge about the mTOR signaling pathway and a short introduction about MAP kinases.

In Chapter 2, we demonstrate a role for MAP4K3 in mTORC1 regulation. In MAP4K3 k.o. cells, we show that mTORC1 is unable to be activated in the presence of amino acids. We hypothesize that MAP4K3 is critical for the activation of mTORC1 through the inhibition of AMPK and TSC2, upstream inhibitors of mTORC1, in the presence of amino acids. We further hypothesize that the inhibition of AMPK by MAP4K3 is through the localization of LKB1. LKB1 is usually localized in the nucleus where its kinase activity towards AMPK is suppressed. In MAP4K3 k.o. cells, we show that there is more LKB1 in the cytosol and this results to more activated AMPK and less Rheb-dependent activation of mTORC1.

Through these complex mechanisms, MAP4K3 emerges as a critical regulator of cellular homeostasis in response to amino acids via mTORC1 activity.

CHAPTER 1

MAP4K3 and the mTOR signaling pathway

mTOR

Mammalian target of rapamycin or mechanistic target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine kinase that belongs to the phosphatidylinositol kinase-related (PIKK) family (Yang et al., 2013). mTOR exists in two distinct complexes, mTORC1 and mTORC2, which consist of different mTOR protein binding partners. mTORC1 has been more widely studied in the field due to its higher sensitivity towards rapamycin as compared to mTORC2 (Jacinto et al., 2004; Loewith et al., 2002). Furthermore, mTORC1 has also been shown to be a master regulator of both catabolic activities like autophagy and anabolic activities such as protein or lipid synthesis (Dibble and Manning, 2013; Howell et al., 2013). mTORC1 is composed of the protein kinase mTOR, regulator-associated protein of mTOR (Raptor) which is involved in substrate recognition, proline-rich Akt substrate- 40kDa (PRAS40), Dep-domain mTOR interacting protein (Deptor), Tti1/Tel2 complex and mammalian lethal with SEC13 protein 8 (mLST8) (Laplante and Sabatini, 2012a; Sarbassov et al., 2004).

mTOR activity is tightly regulated because its downstream targets and functions are critical to maintain cellular homeostasis. The most well characterized downstream target of mTORC1 regulation in cells is the control of mRNA translation (Chauvin et al.,

2014). When active, mTOR stimulates translational initiation through the phosphorylation and suppression of eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), which is an inhibitor of translation. In addition, mTOR also phosphorylates and activates p70 ribosomal S6 Kinase 1 (S6K1), which up regulates the transcription of genes that increase ribosomal biogenesis (Goberdhan et al., 2016; Wang et al., 2001). Several other processes that are also controlled by mTORC1 include lipid synthesis, autophagy, mitochondrial activity and biogenesis (Dibble and Manning, 2013; Howell et al., 2013). Since the downstream processes are costly in terms of cellular energy, it is no surprise that cells have very intricate mechanisms in regulating these pathways.

mTORC1 Inputs

Active mTORC1 has been shown to translocate to the lysosomes where it gets activated by Rheb (Ras homologue enriched in brain) GTPase. Rheb is a small G protein of the Ras superfamily that is involved in transducing growth factor, hormonal signals and cellular energy levels to regulate mTOR (Kim et al., 2008; Long et al., 2005a; Long et al., 2005b; Manning and Cantley, 2003; Vander Haar et al., 2007). Since Rheb is a GTPase, its activity can be regulated by GAPs and GEFs which affect its GTP-binding status (Manning and Cantley, 2003; Roccio et al., 2006). Thus, nutrient sufficiency or stress signals can regulate mTORC1 either directly through its localization or indirectly through the activity of Rheb.

Upstream of mTORC1 are three main inputs – amino acids, growth factors and energy levels – which act in two parallel pathways to influence the activation of mTORC1 (Figure 1.1).

Amino acids, have been shown to activate mTORC1 activity by affecting its subcellular localization (Sancak et al., 2008). Even though the direct amino acid sensors are still unclear and several models of amino acid sensing has been proposed, the Rag GTPases have been identified to be the center of where amino acid signals converge to activate mTORC1 (Bar-Peled et al., 2013; Sancak et al., 2008; Sekiguchi et al., 2001). The Rag GTPases are members of the Ras family of GTP-binding proteins and mammals express 4 different Rag proteins – Rag A, Rag B, Rag C and Rag D. These exist in heterodimers and become active when Rag A/B is GTP-bound and Rag C/D is GDP-bound (Gao and Kaiser, 2006; Kim et al., 2008; Sekiguchi et al., 2001). The Rag GTPase respond to amino acid sufficiency signals by recruiting mTORC1 to the lysosome where Rheb GTPase can activate it (Sancak et al., 2010; Sancak et al., 2008). Since the Rag GTPases are so important for mTORC1's activation, its activity is regulated by both GEF and GAP proteins. The Ragulator complex (LAMTORs 1-5) and GATOR1 complex has been identified to be a GEF and GAP respectively for Rag A/B (Bar-Peled et al., 2013; Bar-Peled et al., 2012).

Growth factors, which include hormones, cytokines and chemokines, activate mTOR by binding receptor tyrosine kinases (RTKs) and G-coupled protein receptors (GPCRs), which activate the PI3K pathway (Vanhaesebroeck et al., 2012). PI3K

generates phosphatidylinositol-3,4,5-triphosphate (PIP_3) which brings 3-phophoinositide-dependent kinase 1 (PDK1) and Akt together to promote the phosphorylation and activation of Akt (Dibble and Cantley, 2015). Activated Akt then phosphorylates and inactivates TSC2, which is present in a heterotrimeric complex with TSC1 and TBC1D7. TSC2 is a GAP for the downstream Rheb GTPase, which causes Rheb to hydrolyze its bound GTP to GDP (Gao et al., 2002; Potter et al., 2002; Zhang et al., 2003). Thus, its inactivation will allow for Rheb to be GTP-bound and active so mTORC1 can be activated by Rheb.

AMP-activated protein kinase (AMPK) is a cellular energy sensor that monitors the ratio of ATP to ADP/AMP levels. (Gowans and Hardie, 2014). Under low energy levels, AMPK is phosphorylated at threonine 172 by liver kinase B1 (LKB1), a tumor suppressor gene that is implicated in Peutz-leghers syndrome (Boudeau et al., 2003). AMPK responds by activating catabolic pathways to produce ATP and down regulates anabolic pathways that are energy expensive. Thus, AMPK phosphorylates and activates TSC2, stimulating TSC2's GAP activity towards Rheb and ultimately inhibiting mTORC1 (Inoki et al., 2002; Inoki et al., 2006).

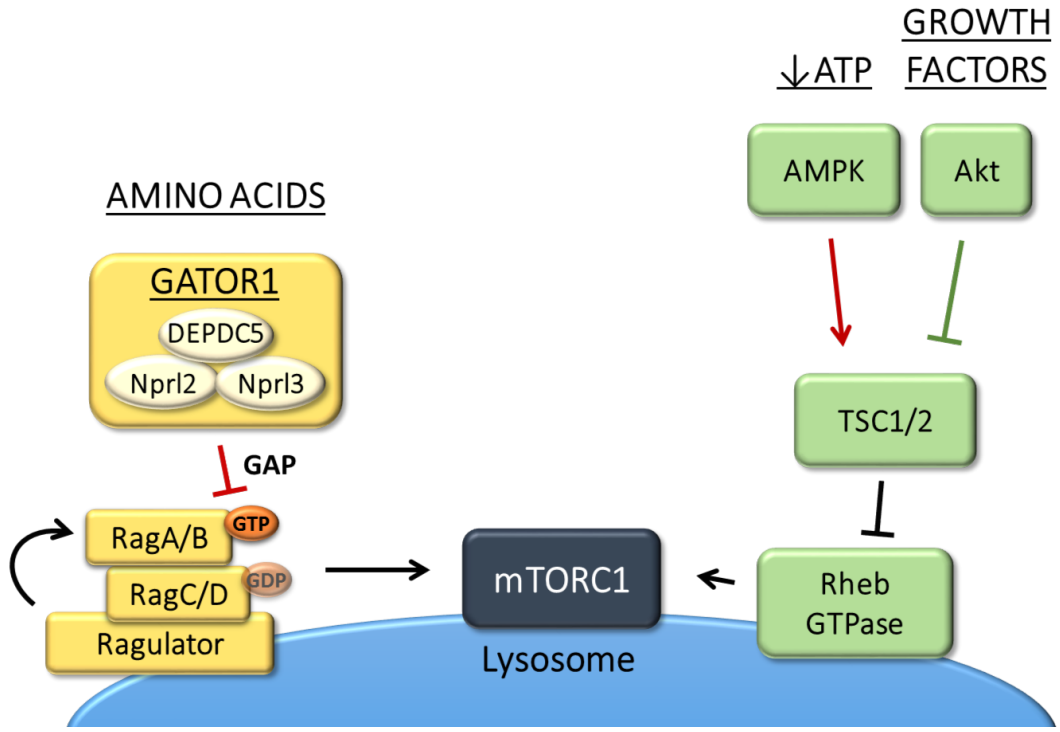

Figure 1.1. Schematic of nutrient signaling to mTORC1 at the lysosome.

MAPKs

The mitogen-activated protein kinases (MAPKs) are protein Ser/Thr kinases that translate extracellular stimuli into a wide variety of cellular response (Gaestel, 2008). They are often activated through phosphorylation of a MAPKK kinase (MAPKKK) after an interaction with a small GTP-binding protein of the Ras/Rho family in response to an extracellular stimulus, which leads to downstream phosphorylation and activation of MAPK Kinase (MAPKK) and the eventual signal transduction to MAPK (Robbins et al., 1993). All eukaryotic cells possess multiple MAPK pathways and their phosphorylation is critical to regulate diverse cellular processes such as cell proliferation, differentiation, stress response, and even apoptosis (Avruch, 2007; Cargnello and Roux, 2011). The

multiple tiers of phosphorylation by various MAP kinases provide fine-tuning to the signaling cascade and offer a tighter regulation in its signal transduction.

MAP4K3 and the mTOR signaling pathway

MAP4K3, also known as germinal center kinase-like kinase (GLK), is a member of the Ste20 family of protein kinases. MAP4K3 has been shown to play an important role in the nutrient regulation of mTOR signaling. It has been shown that MAP4K3 is required for amino acids but not insulin to induce phosphorylation of mTORC1's downstream targets, S6K and 4EBP1 (eIF4E-binding protein) (Findlay et al., 2007). Following amino acids withdrawal, Ser170 on MAP4K3 is dephosphorylated via PP2A phosphatase, resulting in its inactivation and inhibition of mTORC1 signaling (Yan et al., 2010). Consistent with these findings, *Drosophila* flies lacking MAP4K3 also show reduced TORC1 activity, smaller body size, and reduced growth rate, which closely resemble flies that are starved (Bryk et al., 2010). These findings reinforce MAP4K3's role in the regulation of mTORC1 signaling in response to amino acids.

CHAPTER 2

MAP4K3 regulates mTORC1 through LKB1 and AMPK

Abstract

The mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) is a master regulator that integrates multiple nutritional and environmental cues such as amino acid and growth factors availability, cellular energy status and stress to promote cell growth or autophagy. Over the past decade, a lot of attention has been focused on elucidating the amino acid signaling pathway that activates mTORC1. The field acknowledges a role of mitogen activated protein 4 kinase 3 (MAP4K3) in the amino acid dependent activation of mTORC1 but its exact mechanism is not understood. Here, we propose a mechanism by which MAP4K3 suppresses upstream inhibitors such as AMPK and TSC2 in the presence of amino acids to activate mTORC1. We hypothesize that MAP4K3 inhibits AMPK by regulating LKB1 localization and hence its kinase activity towards AMPK. In the presence of amino acids, MAP4K3 allows for the retention of LKB1 in the nucleus where it is separated from cytosolic AMPK. Thus, AMPK is less active and TSC2 GAP activity towards Rheb is suppressed, allowing for mTORC1 activation. Through this complex mechanism, MAP4K3 is able to tightly regulate the activity of mTORC1, allowing for precise regulation of cell cycle and metabolism in the presence of amino acids.

Introduction

Cells in multicellular organisms grow in response to intrinsic cell signals such as nutrients or growth factors availability. The ability to properly integrate these signals of nutrient starvation or sufficiency to coordinate with cell proliferation is of utmost importance and the pathways that relay these signals are often dysregulated in human diseases such as cancer or diabetes.

The mechanistic target of rapamycin (mTOR) is a highly conserved serine/threonine kinase where most nutrient or stress signals converge so that mTOR can respond accordingly by regulating protein synthesis and cell growth (Dibble and Manning, 2013; Laplante and Sabatini, 2012b). A lot of work has been done to elucidate the upstream metabolic signaling pathways of mTORC1 and two pathways that act in parallel to control mTOR activity has been identified, both of which ultimately end with two classes of Ras-related small G proteins lying upstream of mTORC1.

The first class of G proteins, the Rag GTPases, function as a heterodimer of RagA/RagB complexed with RagC/RagD (Sancak et al., 2008). The Rag heterodimers are tethered to the lysosomes by binding to the Ragulator (Sancak et al., 2010). In response to amino acids availability, RagA/B becomes GTP-loaded through the action of Ragulator's guanine nucleotide exchange factor (GEF) ability (Bar-Peled et al., 2012) while Rag C/D becomes GDP-loaded through an unknown mechanism. Adopting this conformation, the Rag heterodimers move to recruit mTORC1 to the lysosomal surfaces

through their interaction with Raptor (Bar-Peled et al., 2012; Sancak et al., 2010). This recruitment, however, is insufficient for mTOR activation. (Sancak et al., 2010).

mTOR requires the second class of G proteins, the Rheb GTPase, for its activation (Long et al., 2005a; Long et al., 2005b). Rheb has been shown be localized to the lysosomes and its GTP/GDP status is controlled by energy levels and the presence of growth factors (Dibble and Cantley, 2015). Rheb activity, dictated by its GTP loading status, is controlled by the tuberous sclerosis complex (TSC). The TSC complex is composed of three main proteins – TSC tumor suppressor 1 (TSC1), TSC tumor suppressor 2 (TSC2) and Tre2-Bb2-Cdc16-1 domain family member 7 (TBC1D7) (Inoki et al., 2003; Manning and Cantley, 2003; Tee et al., 2002). Within the TSC complex, TSC2 acts as the GAP for Rheb while TSC 1 is a scaffolding protein and TBC1D7 stabilizes the complex (Potter et al., 2002; Smith et al., 2005).

TSC2 has been shown to be necessary to transduce signals regarding cellular energy status, growth factors and insulin. Insulin and growth factors activate mTORC1 by deactivating TSC2 through the stimulation of the class 1 phosphatidylinositol-3-kinase (PI3K) and the downstream effector Akt, a protein kinase that activates TSC2 (Dibble and Cantley, 2015; Garami et al., 2003; Manning et al., 2002). The phosphorylation of TSC2 by Akt has been shown to inhibit TSC2's GAP activity towards Rheb (Inoki et al., 2002; Tee et al., 2003). Thus, insulin and growth factors signaling negatively regulates the TSC complex to allow for Rheb-dependent activation of mTORC1.

Cellular energy levels are sensed by a separate upstream pathway involving AMP-activated protein kinase (AMPK). AMPK is a heterotrimeric protein that is comprised of the catalytic α subunit, a β and γ subunit. The γ subunit is able to bind to adenosyl nucleotides such as ATP, ADP or AMP. AMPK activity is suppressed when its γ subunit is bound to ATP. In situations where the cell undergoes stress, cellular AMP levels become higher and AMPK becomes activated when the γ subunit is bound to AMP (Gowans and Hardie, 2014; Hawley et al., 2003; Mihaylova and Shaw, 2011). Activated AMPK can signal to mTORC1 by phosphorylating and activating TSC2 GAP activity to prevent Rheb-dependent activation of mTORC1 or by phosphorylating and inhibiting Raptor, a component of the mTORC1 complex. (Gwinn et al., 2008). AMP binding also promotes the upstream kinase liver kinase B1 (LKB1) to be complexed with STRAD and MO25, allowing for LKB1 to phosphorylate AMPK at Thr172. This phosphorylation increases AMPK's activity almost 100 fold (Hawley et al., 2003; Lizcano et al., 2004; Shaw et al., 2004; Suter et al., 2006). In summary, while Akt activates mTORC1 by suppressing TSC2, AMPK deactivates mTORC1 by activating TSC2.

Previously, MAP4K3 was identified as a Ste20 kinase that regulates the activity of mTORC1 in response to amino acids but not insulin or rapamycin (Findlay et al., 2007). However, the mechanism of how MAP4K3 regulates mTORC1 has not yet been elucidated. Here, we demonstrate that MAP4K3 is required for mTORC1 activation in the presence of amino acids and we hypothesize that MAP4K3 regulates mTORC1 upstream of Rheb. We further hypothesize that MAP4K3 is regulating mTORC1 by affecting the subcellular localization of LKB1 and the inhibition of AMPK and TSC2, to

allow for Rheb-dependent activation of mTORC1. Through this complex mechanism, MAP4K3 is able to tightly regulate the activity of mTORC1 based on the presence of amino acids.

Results

MAP4K3 is necessary for mTORC1 activation in the presence of amino acids

In order to study the role of MAP4K3 in mTORC1 activation, we made complete MAP4K3 knockout (k.o.) cells. We performed CRISPR-Cas 9 genome editing in Wild Type (WT) HEK293A (Human Embryonic Kidney) cells with guide sequences targeting two different exon sequences of MAP4K3 and obtained two distinct clones (M1 and M4) with frame shift mutations at either of the two targeted sites. We confirmed the absence of MAP4K3 protein expression in these two cell lines by western blot analysis (Figure 2.1).

We then tested mTORC1 activation in WT and MAP4K3 k.o. cells in response to the presence and absence of amino acids. By probing for the phosphorylation of downstream substrates of mTORC1 such as S6 Kinase (S6K), eIF4E (eukaryotic initiation factor 4E)- binding protein 1 (4EBP1) and ribosomal protein S6, we identified a gradual reduction in the phosphorylation of mTORC1 target substrates upon amino acid withdrawal and a sharp increase in their phosphorylation upon amino acid re-stimulation for 10 minutes in wild-type cells (Figure 2.2). While there is no difference in the mTORC1 activation response to amino acid starvation in MAP4K3 k.o. cells, there is substantial difference after just 10 minutes of amino acid re-stimulation. MAP4K3 k.o. cells showed a lack of phosphorylation in S6K, S6 and 4EBP1 in response to amino acid stimulation. Since mTORC1 promotes cell growth in response to nutrients like amino acids, we performed an assay to measure growth rate and cell size of our MAP4K3 k.o.

cells. Consistent with the results from our immunoblot, MAP4K3 k.o. cells exhibited a significantly slower growth rate after day 2 (Figure 2.3) and smaller cell size as compared to WT cells (Figure 2.4). These results support the important role of MAP4K3 in activating mTORC1 in the presence of amino acids.

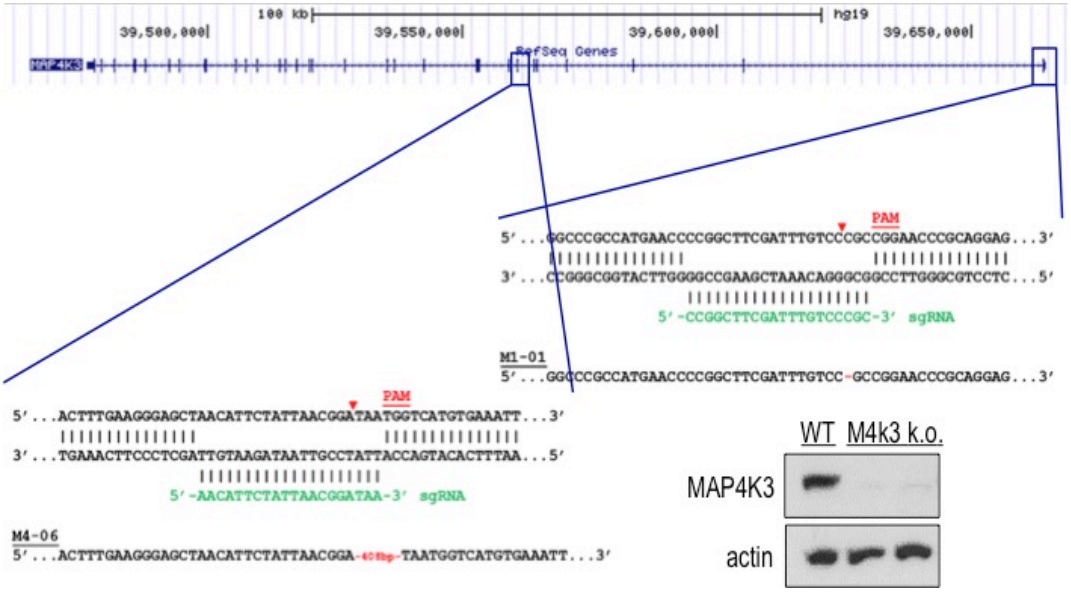

Figure 2.1. Validation of MAP4K3 k.o. cells. WT and MAP4K3 k.o. cell lysates were validated for MAP4K3 knock out via immunoblot

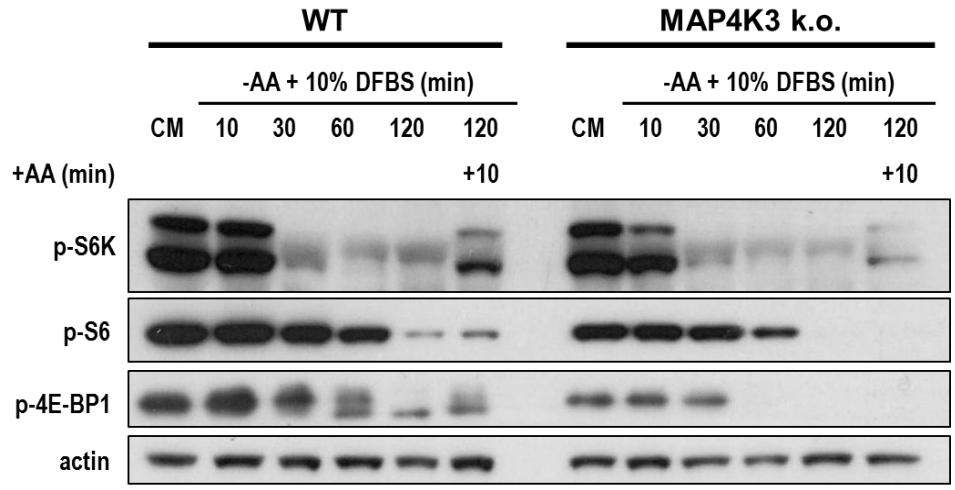

Figure 2.2. MAP4K3 is necessary for mTORC1 activation by amino acids. WT and MAP4K3 k.o. cells were starved for 10, 30, 60, and 120 minutes, then re-stimulated with amino acids for 10 minutes. Cell lysates were immunoblotted for the phosphorylation state of S6K1, S6, and 4E-BP1.

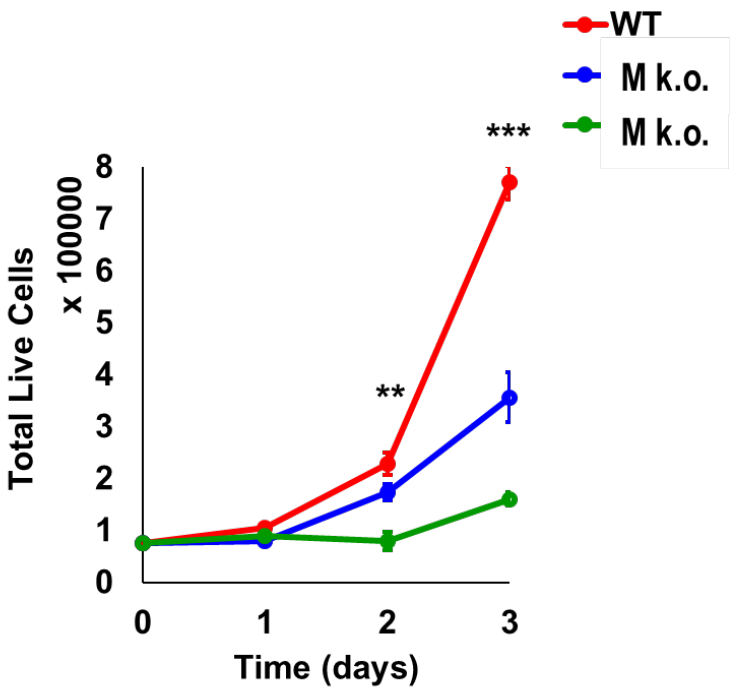

Figure 2.3. MAP4K3 is important for maintaining cellular growth. Cells were grown in normal media conditions.

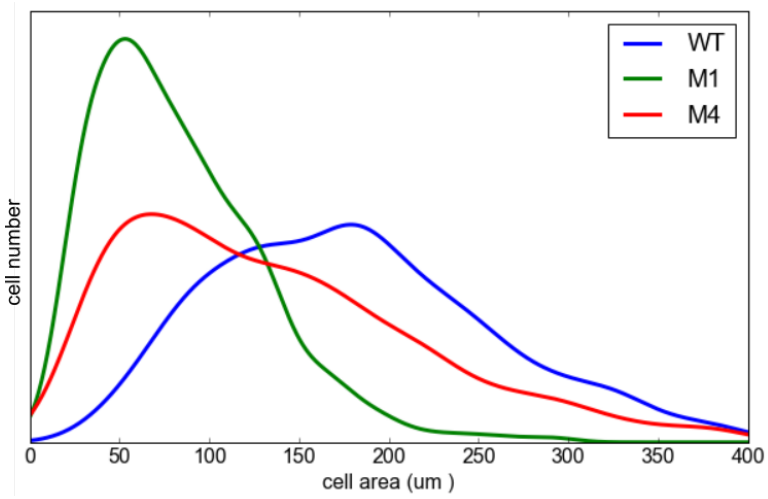

Figure 2.4. MAP4K3 is important for maintaining cell size. Kernel density plot of cell size of WT cells and two MAP4K3 k.o. cell lines grown in complete media.

MAP4K3 signals upstream of Rheb GTPase to activate mTORC1

In response to amino acids, mTORC1 is recruited to the lysosomes where it is in close proximity with Rheb (Ras homologue enriched in brain) GTPase (Resnik-Docampo and de Celis, 2011). Since Rheb is needed to activate mTORC1, we hypothesize that MAP4K3 could be affecting the interaction between mTORC1 and Rheb. Rheb is a small G protein of the Ras superfamily that influences cell growth through the activation of mTORC1. To test this, we overexpressed HA-tagged Raptor, a component of the mTORC1 complex and myc-tagged Rheb GTPase and performed a co-immunoprecipitation experiment to observe differences in their interaction. To our surprise, mTORC1 interacted more strongly with Rheb in MAP4K3 k.o. cells as compared to WT cells (Figure 2.5 and 2.6). This effect was observed in both reciprocal pull-downs. This data may seem contradictory since increased mTORC1 interaction with Rheb should be accompanied by increased mTORC1 activity in MAP4K3 k.o. cells, not decreased mTORC1 activity. However, it is understood that their interaction is GTP status dependent, and only the GTP-bound Rheb can activate mTOR (Long et al. 2005). Thus, even when there is more interaction, mTOR could still be inactive if Rheb is bound to GDP.

We then proceeded to investigate the activity of Rheb in MAP4K3 k.o. cells. We incubated cell lysates with immobilized γ-Aminohexyl-m^7GTP on agarose beads and immunoblotted for Rheb. Indeed, there is less GTP bound Rheb in the MAP4K3 k.o. cells (Figure 2.7). Rheb is a GTPase and its activity is regulated by TSC2, which is a GAP for Rheb (Garami et al., 2003; Zhang et al., 2003). Consistent to the binding preference of

many GAPs for the GTP-loaded state of target GTPases, TSC2 preferentially interacts with GTP-bound Rheb (Carroll et al., 2016). We identified a stronger interaction between endogenous TSC2 and Rheb in the WT cells as compared to the MAP4K3 k.o. cells (Figure 2.8). These findings suggest that there is lesser GTP-loaded Rheb in MAP4K3 k.o. cells as compared to the WT cells. When there is lesser active Rheb, the amount of active mTORC1 will also decrease. In addition, overexpression of constitutively active (CA) Rheb Q64L mutant was sufficient to rescue the dampened mTORC1 response in the MAP4K3 k.o. cells following amino acid re-stimulation (Figure 2.9). CA Rheb was even able to constitutively activate mTORC1 signaling in both WT and MAP4K3 k.o. cells regardless of amino acid presence. These results suggest that MAP4K3 may activate mTORC1 through the activation of Rheb.

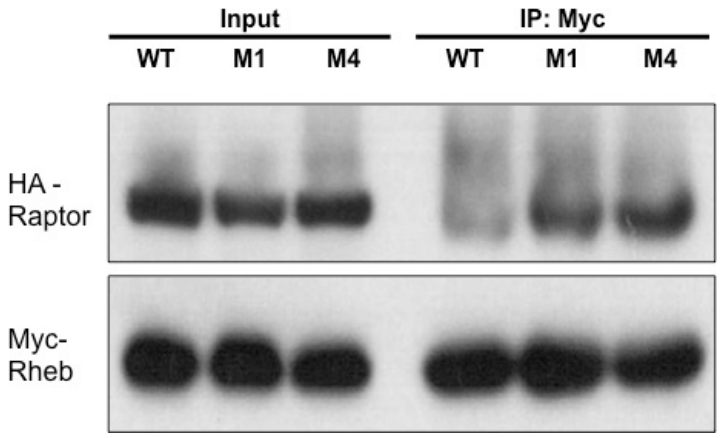

Figure 2.5. MAP4K3 k.o. cells have more interaction between Rheb and Raptor. Lysate were prepared from WT and two MAP4K3 k.o. cell lines over expressing HA-Raptor and Myc-Rheb. Samples were immunoprecipitated with Myc antibody and immunoblotted with HA antibody for Raptor.

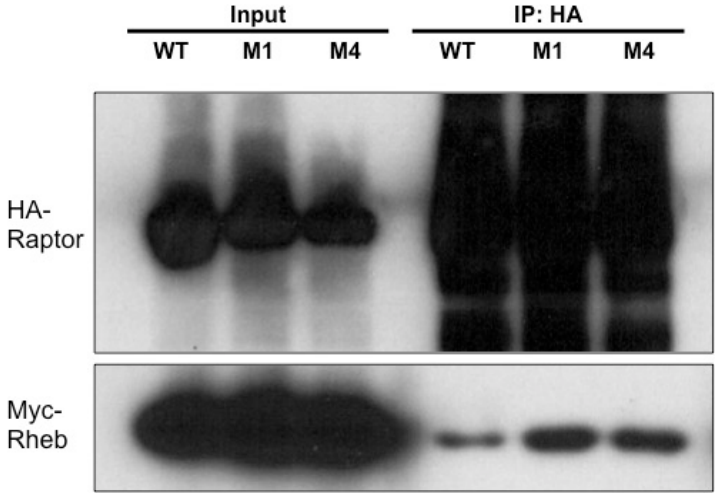

Figure 2.6. MAP4K3 k.o. cells have more interaction between Rheb and Raptor in reciprocal Co-IP. Lysate were prepared from WT and two MAP4K3 k.o. cell lines over expressing HA-Raptor and Myc-Rheb. Samples were immunoprecipitated with HA antibody and immunoblotted with Myc antibody for Rheb.

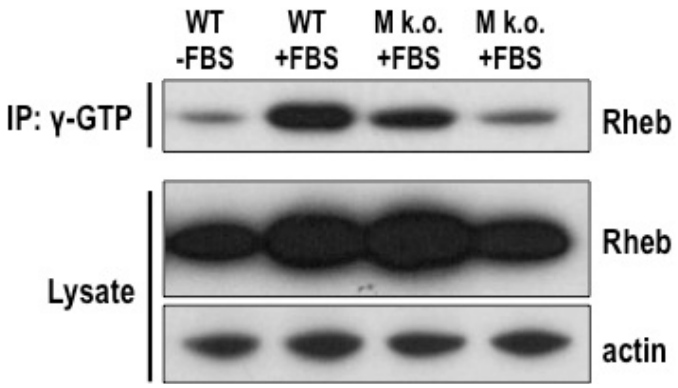

Figure 2.7. MAP4K3 k.o. cells have less GTP-bound Rheb. Lysate were prepared from WT and two MAP4K3 k.o. cell lines in complete DMEM with or without 10% Fetal Bovine Serum (FBS). Cell lysates were incubated with immobilized γ-Aminohexyl-m^7GTP on agarose beads, processed and immunoblotted for Rheb.

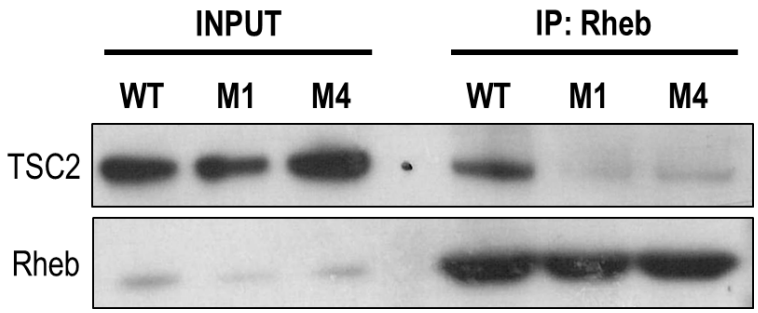

Figure 2.8. Loss of MAP4K3 leads to a decrease in endogenous GTP-bound Rheb. Lysate were prepared from WT and two MAP4K3 k.o. cell lines and immunoprecipitated with Rheb antibody for endogenous TSC2.

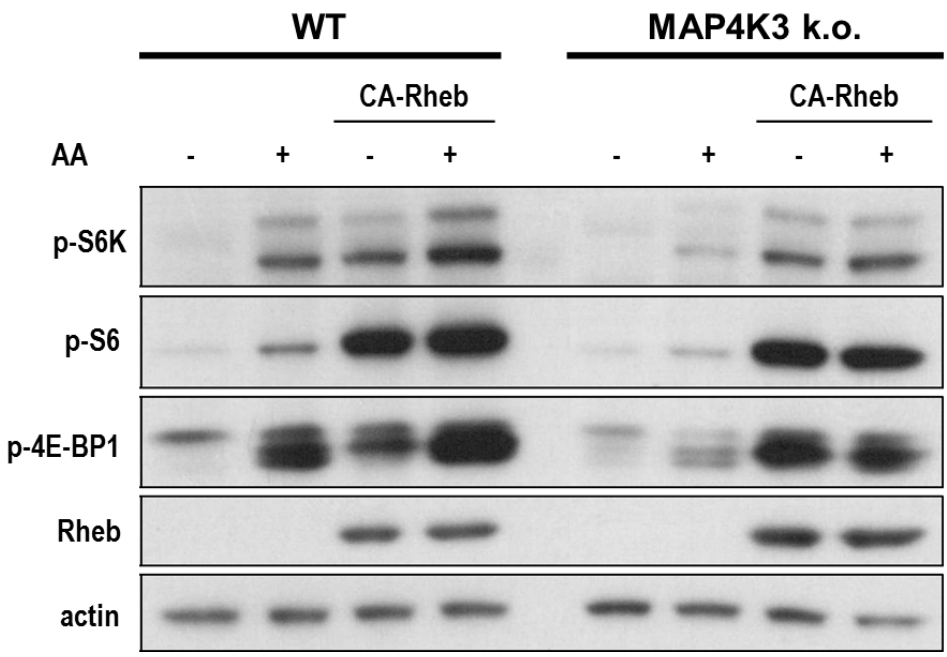

Figure 2.9. MAP4K3 regulates mTORC1 activation upstream of Rheb GTPase. WT and MAP4K3 k.o. cells were transfected with constitutively active Rheb where indicated and starved of amino acids for 3 hours or starved and re-stimulated with amino acids for 10 minutes. Lysates were prepared and immunoblotted for the indicated proteins.

MAP4K3 regulates Rheb GTPase via AMPK and TSC2

TSC2 has been shown to be a key regulator in mTORC1 activity in response to cellular feedback on energy and stress signals (Inoki, K. et al. 2003b; Huang, J. and Manning et al. 2008; Liu, L. et al. 2006). As Rheb is regulated by TSC2, which affects its GTP loading and hence its activity, we went on to investigate upstream regulators of TSC2. TSC2 has been well characterized to play a role in insulin/growth factor sensing via the phosphoinositide 3-kinase/Akt pathway as well as the AMPK pathway in response to high AMP/ADP levels (Inoki, K. et al., 2006; Manning et al., 2002). To identify which pathway was responsible for the increased TSC2 activity, we immunoblotted for phosphorylated AMPK and Akt in the MAP4K3 k.o. cells. While there is no difference in p-Akt levels between the MAP4K3 k.o. cells as compared to the WTs (Figure 2.10), we observed more phosphorylated AMPK in the MAP4K3 k.o. cells when they are re-stimulated with amino acids (Figure 2.11). In addition, AMPK was revealed to interact with MAP4K3 through mass spectrometry. It appears that MAP4K3 might be activating mTORC1 in response to amino acids by repressing targets that down-regulate mTORC1 activity. To confirm that MAP4K3 is acting upstream of AMPK, we made MAP4K3/AMPK double k.o. cells using CRISPR/Cas9 genome editing. While the MAP4K3 k.o. cells show a dampened mTORC1 response to amino acids stimulation, the MAP4K3/AMPK double k.o. cells show a rescue of mTORC1 levels back to levels comparable to the WT cells (Figure 2.11). In support of our hypothesis, we saw a rescue in the slower cell growth as previously seen in MAP4K3 single k.o. cells. (Figure 2.12).

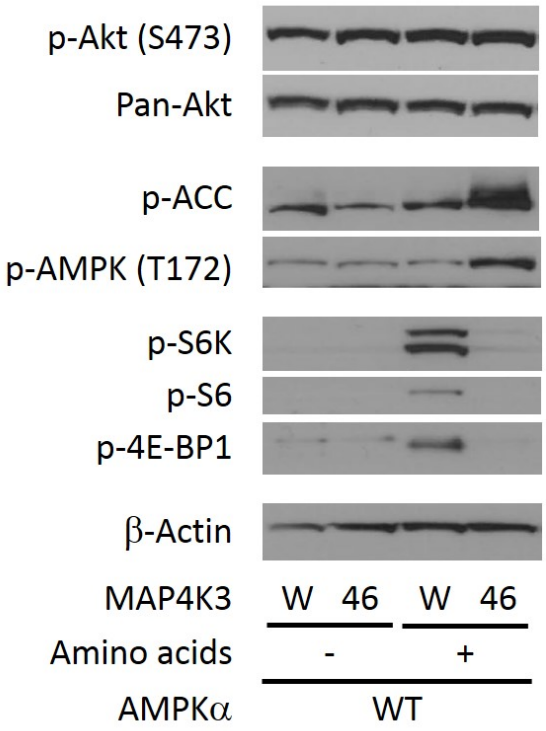

Figure 2.10. MAP4K3 regulates mTORC1 indepedently of the Akt pathway. WT, MAP4K3 k.o. cells were amino acid starved for 3 hours or starved and re-stimulated with amino acids for 30 minutes. Lysates were prepared and immunoblotted for the indicated proteins.

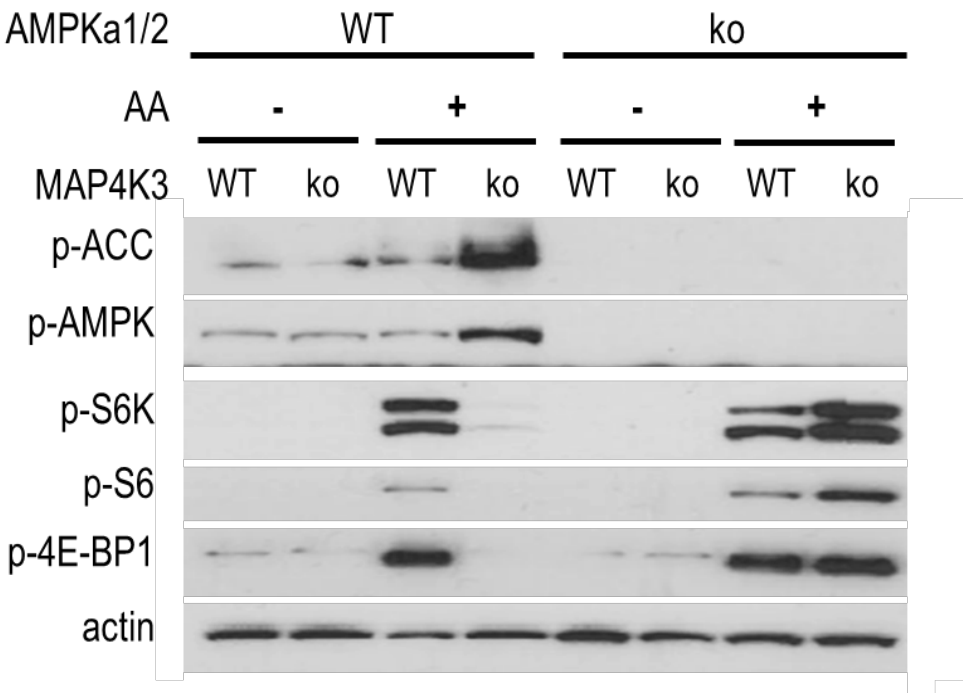

Figure 2.11. MAP4K3 regulates mTORC1 activation upstream of AMPK. WT, MAP4K3 single k.o., AMPK single k.o., and MAP4K3/AMPK double k.o. cells were amino acid starved for 3 hours or starved and re-stimulated with amino acids for 30 minutes. Lysates were prepared and immunoblotted for the indicated proteins.

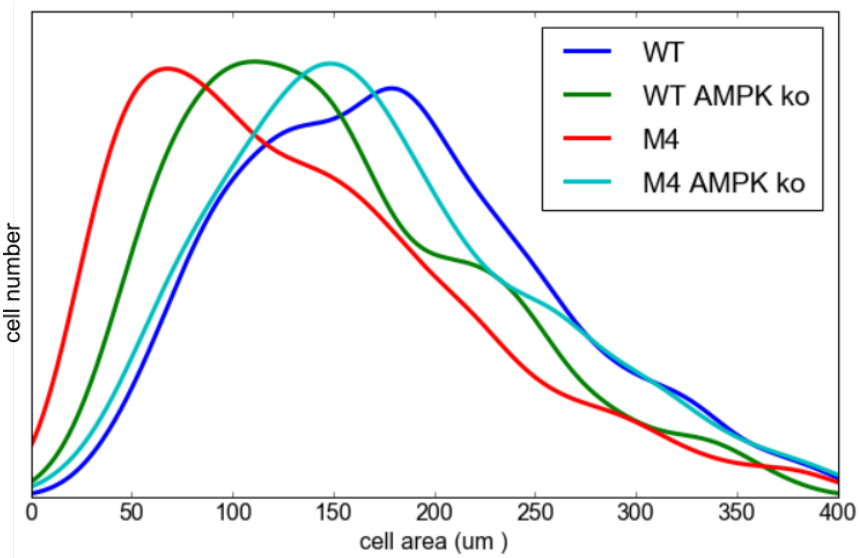

Figure 2.12. MAP4K3 regulates mTORC1 activation upstream of AMPK. Kernel density plot of cell size of WT, MAP4K3 single k.o., AMPK single k.o., and MAP4K3/AMPK double k.o. cell lines grown in complete media.

MAP4K3 regulates AMPK activity through LKB1

The AMP-activated protein kinase (AMPK) is a master regulator of metabolic homeostasis by responding to cellular energy status (Zhang et al., 2013). AMPK is mainly activated via phosphorylation by the upstream liver kinase B1 (LKB1) upon energy preservation (Hawley et al., 2003; Lizcano et al., 2004). Hence, we asked the question if MAP4K3 is regulating AMPK through LKB1. To answer this, we made MAP4K3/LKB1 double k.o. cells using CRISPR-Cas9 genome editing. Similar to the results we observe in the MAP4K3/AMPK double k.o. cells, the MAP4K3/LKB1 double k.o. cells rescued the dampened mTORC1 inactivation phenotype we observed in the MAP4K3 single k.o. cells (Figure 2.13). Furthermore, the MAP4K3/LKB1 knockouts showed an improvement in their reduced growth rates as seen in the MAP4K3 k.o. cells (Figure 2.14). These results indicate that MAP4K3 should be signaling upstream of LKB1-AMPK axis to activate mTORC1.

LKB1 activity is determined by its nuclear or cytoplasmic localization. LKB1 is normally found in the nucleus in its inactive state but it gets activated when STRAD moves into the nucleus to recruit LKB1 out to the cytosol where LKB1 forms a trimeric complex with STRAD and Mo25 (Boudeau et al., 2004; Dorfman et al., 2008). Thus, we assayed for LKB1 localization with microscopy in our WT and MAP4K3 k.o. cells. In line with our hypothesis, we found that LKB1 is predominantly cytosolic in our MAP4K3 k.o. cells after amino acid restimulation whereas most of the LKB1 in the WT cells returned to the nucleus (Figure 2.15). This result has also been replicated with endogenous LKB1 by immunoblot of LKB1 in the respective nuclear and cytoplasmic

fractions (Figure 2.16). This is indicative of more active LKB1 in the MAP4K3 k.o. cells following amino acid restimulation and more phosphorylated AMPK, which ultimately results in lesser mTOR activation.

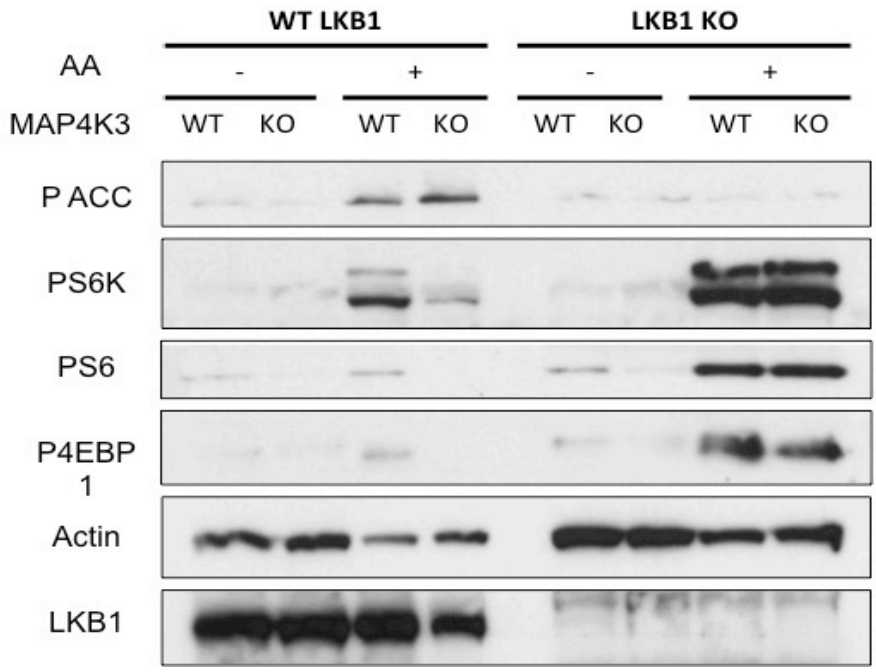

Figure 2.13. MAP4K3 regulates mTORC1 activation upstream of LKB1. WT, MAP4K3 single k.o., LKB1 single k.o., and MAP4K3/LKB1 double k.o. cells were amino acid starved for 3 hours or starved and re-stimulated with amino acids for 10 minutes. Lysates were prepared and immunoblotted for the indicated proteins.

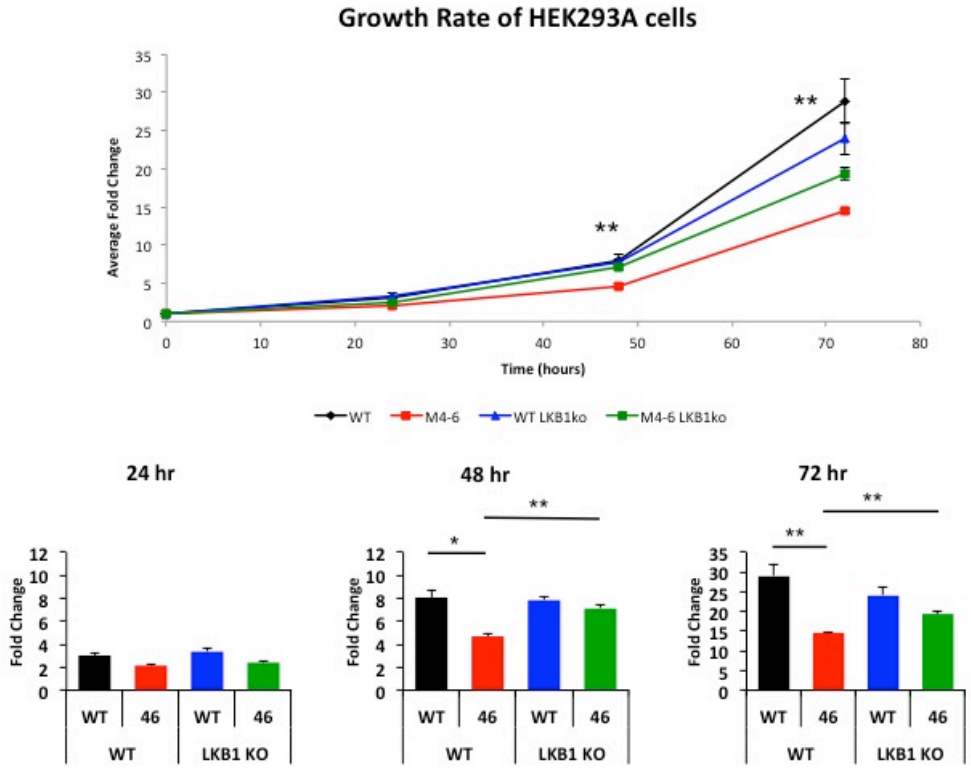

Figure 2.14. MAP4K3 regulates mTORC1 activation upstream of LKB1. WT, MAP4K3 single k.o., LKB1 single k.o., and MAP4K3/LKB1 double k.o. cell lines grown in complete media.

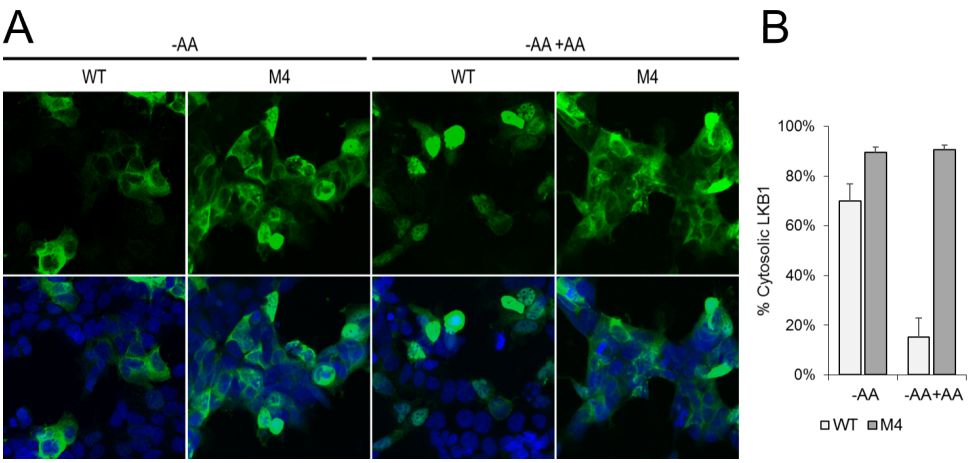

Figure 2.15. MAP4K3 regulates LKB1 subcellular localization. (A) WT and MAP4K3 k.o. cells were transfected with LKB1-FLAG, then amino acid starved for 3 hours or starved and re-stimulated with amino acids for 30 minutes, before immunostaining for LKB1-FLAG (green) and DAPI (blue). **(B)** Quantification of the percentage of LKB1 that is localized in the cytosol in (A).

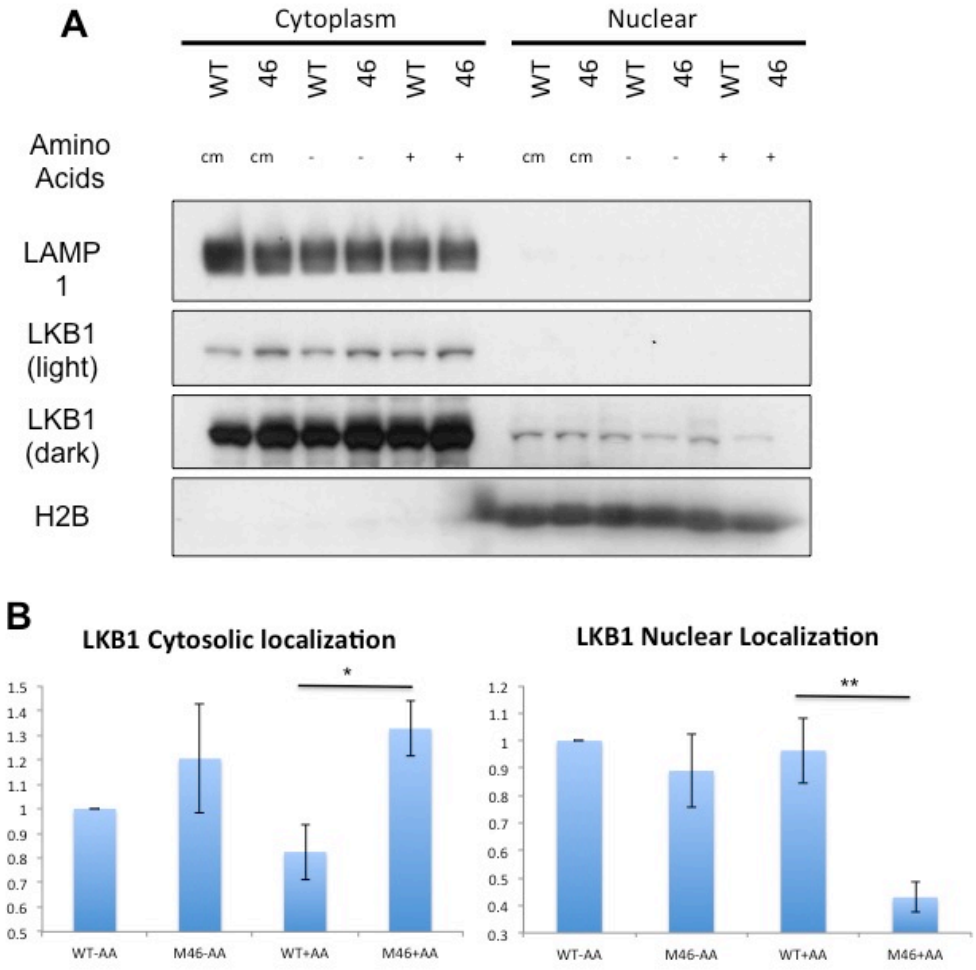

Figure 2.16. MAP4K3 regulates LKB1 subcellular localization. (A) WT and MAP4K3 k.o. cells were amino acid starved for 3 hours or starved and re-stimulated with amino acids for 30 minutes, before subcellular fractionation and immunoblotted for the indicated proteins. **(B)** Quantification of the fold change of LKB1 in various conditions normalized to WT starved with amino acid

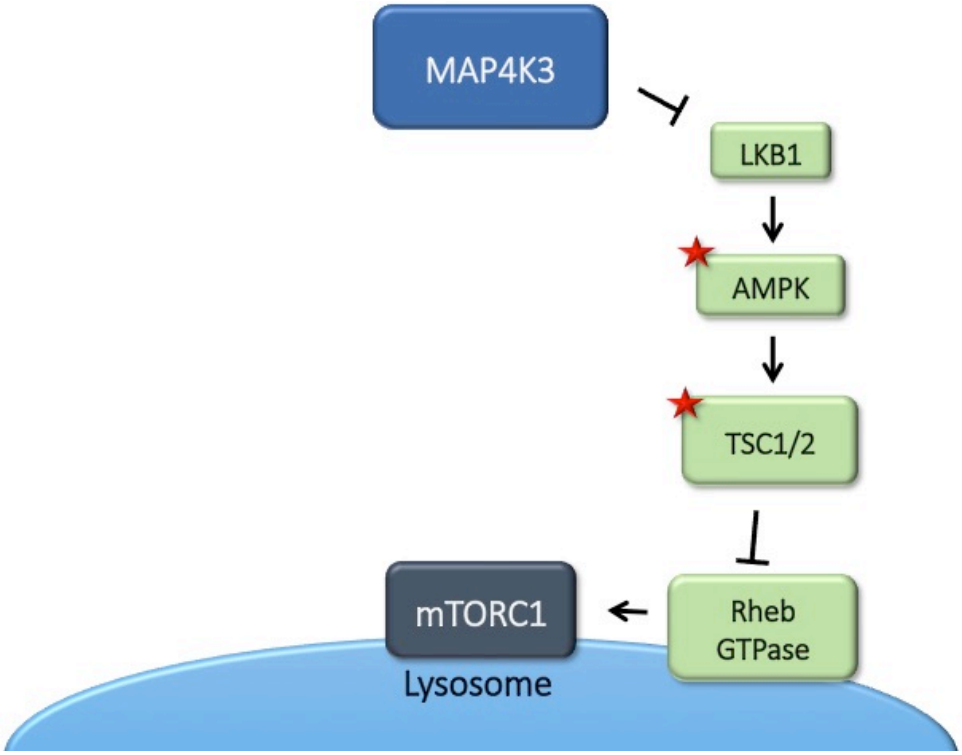

Figure 2.17. Proposed model for MAP4K3 signaling to mTORC1.

Discussion

The mTORC1 signaling pathway is a highly conserved pathway that integrates upstream signals such as nutrients and cellular stress to control cells' metabolic profiles. Previously, amino acids have been known to regulate mTORC1 through the Rags and Ragulator by recruiting mTORC1 to the lysosomes for Rheb dependent activation (Bar-Peled et al., 2012; Sancak et al., 2010). Other studies have hinted that amino acids could signal through TSC2 and Rheb (Carroll et al., 2016; Long et al., 2005b; Roccio et al., 2006), but its exact pathway was never understood. Here, we show that MAP4K3 is necessary to activate mTORC1 signaling in the presence of amino acids, consistent with data published by Findlay et al., and that MAP4K3 is crucial for normal cellular growth rate (Bryk et al., 2010). In MAP4K3 k.o. cells, we observed less mTORC1 activation and slower growth rate as compared to WT cells. Instead of mTORC1 having less interaction with Rheb, the reduced mTORC1 activation is attributed to decreased active GTP-bound Rheb. Thus, we hypothesize that amino acids also activate mTORC1 through Rheb. Working through the upstream regulators of Rheb, we discovered a novel role for MAP4K3 to regulate mTORC1 activity in response to amino acids via the AMPK-TSC2 pathway (Figure 2.17). Our proposed mechanism suggests that MAP4K3 regulates LKB1 localization to suppress AMPK and TSC2, thus enabling mTOR activation. Our results, which show a higher proportion of LKB1 localized in the cytosol and more phosphorylated and activated AMPK in MAP4K3 k.o. cells, support this hypothesis. However, more work still has to be done to identify the exact mechanism by which MAP4K3 affects LKB1 subcellular localization.

Since MAP4K3 is a kinase, it may be possible that LKB1 regulation by MAP4K3 is direct and that LKB1 subcellular localization can be controlled by phosphorylation. However, an indirect regulation of LKB1 by MAP4K3 is certainly possible. Studies have shown that SIRT1 is a key regulator of LKB1 and AMPK activity. For example, Lan et al. demonstrated that overexpression of SIRT1, a class III NAD+-dependent histone/protein deacetylase, leads to increased LKB1 deacetylation, cytoplasmic localization of LKB1, and kinase activity against targets AMPK (Lan et al., 2008). Suchankova et al. showed that SIRT1 inhibitors downregulated the activities of both AMPK and SIRT, while SIRT1 activators increased both proteins' activities (Suchankova et al., 2009). LKB1 localization has also been shown to be affected by the orphan nuclear receptor Nur77 that binds and sequesters LKB1 in the nucleus and hence attenuating AMPK activation (Zhan et al., 2012). Further, STRAD, which is a pseudo kinase, has been shown to be responsible for the recruitment of LKB1 to the cytosol where it forms a trimeric complex with STRAD and MO25 to increase AMPK activity extensively (Dorfman et al. 2008). All of these are possible candidates that may be the bridge to the current signaling gap between MAP4K3 and LKB1.

In summary, we have uncovered MAP4K3 as an important upstream regulator of mTORC1 that controls the activity of Rheb in response to amino acids. In MAP4K3 k.o. cells, we saw phenotypes similar to dysregulated and hyper-active LKB1, which result in increased AMPK and TSC2 activation. Since mTORC1 is implicated in lifespan extension and often dysregulated in human diseases such as cancer and diabetes, we

anticipate that further investigation into the upstream or downstream targets of MAP4K3 can provide greater insight into possible therapies against the pathological effects of mTORC1 pathway dysregulation.

Chapter 2 is an original document describing scientific work that is currently being prepared as a manuscript for submission in a much-revised form. Hsu, C.L., Ohnishi, K., Lee, E.X., Meisenhelder, J., Paz, E.P., Hunter, T., and La Spada, A.R. "MAP4K3 regulates mTORC1 activity via AMPK signaling." The dissertation author is the co-author of this work.

MATERIALS AND METHODS

Cell culture

HEK293A and HeLa cells were grown in DMEM media with 10% FBS. For amino acid deprivation (AAD), cells were treated with Earle's balanced salt solution (EBSS). For autophagic flux determination, Neuro2a cells were co-transfected with the mCherry-GFP-LC3 vector using Lipofectamine 2000, according to the manufacturer's instructions (Invitrogen). After 24 hrs, the media was replaced.

Cell lysis and immunoprecipitation

Cells were rinsed twice with ice-cold PBS and lysed in ice-cold lysis buffer (25mM HEPES-KOH pH 7.4, 150mM NaCL, 5mM EDTA, 1% Triton X-10040 mM, one tablet of EDTA-free protease inhibitors (#11873580001 from Roche) per 10 mL of lysis buffer, and one tablet of PhosStop phosphatase inhibitor, as necessary. The soluble fractions from cell lysates were isolated by centrifugation at 8,000 rpm for 10 mins in a microfuge. Protein lysates were quantified using Pierce BCA Protein Assay Kit (Thermo Scientific) following the manufactures protocol. For immunoprecipitations, primary antibodies were incubated with Dynabeads® (Invitrogen) overnight, then washed with sterile PBS. Antibodies bound to Dynabeads were then incubated with lysates with rotation for 2 hours at 4°C. Immunoprecipitates were washed three times with lysis buffer. Immunoprecipitated proteins were denatured by the addition of 20 µl of sample buffer and boiling for 10 minutes at 70°C, resolved by SDS-PAGE, and analyzed via Western blot analysis.

Western Blot analysis

After SDS-PAGE, proteins were transferred to a 0.45 mm PVDF Immobilon-P membrane (EMD Millipore), and blocked for 1 hr at RT with 5% PBS-T milk. Membranes were incubated overnight with primary antibodies against: LC3 (Novus Biologicals, #NB100-2331) 1/3000; beta actin (abcam, #ab8226) 1/10000; Map4k3 (Cell Signaling #9613) 1/1000; RagA (Cell Signaling #4357) 1/1000; RagC (Cell Signaling #5466) 1/1000; and Lamtor1 (Cell Signaling #8975) 1/1000 in 5% PBST BSA. Antibodies and conditions for immunoblotting of mTOR, phospho-mTOR (S2448), S6K1, phospho-S6K1 (T421/S424), S6RP, and phospho-S6RP (S240/244) have already been described (Dubinsky 2014). Species-specific secondary antibodies were goat anti-rabbit IgG-HRP (Santa Cruz, #sc-2004) or goat anti-mouse IgG-HRP (Santa Cruz, #sc-2005), diluted 1/10,000 in 2% PBS-T milk and incubated for 1 hr at RT. Chemiluminescent signal detection was captured using Pierce ECL Plus Western Blotting Substrate (Thermo Scientific), and autoradiographic film, using standard techniques. Densitometry analysis was performed using ImageJ.

Immunocytochemistry

Cells were seeded in CC2-coated 8-chamber slides (Thermo Fisher) two days prior to experimentation and transfected as indicated. PBS-MC (1mM $MgCl_2$, 0.1mM $CaCl_2$, in PBS) was used for all washes and as a diluent for all solutions. Cells were fixed with 4% paraformaldehyde in PBS-MC for 12 minutes, then washed 3 times. Then 0.05% Triton-X in PBS-MC was used to permeabilize the cells for 5 minutes, followed by 2

washes in PBS-MC. Primary and secondary antibodies were diluted in 5% normal goat serum in PBS-MC. Cells were incubated in primary antibodies for 2 hours at room temperature, followed by 4 washes in PBS-MC. Cells were incubated in secondary antibodies for 1 hour at room temperature. Cells were washed 4 times in PBS-MC, then mounted with Prolong gold antifade reagent with DAPI (#P-36931 from Invitrogen). Images were captured with a Zeiss LSM 780 confocal microscopy and analyzed with Zen 2011 LSM 780 software and Image J.

GTP Binding Assay

For binding of Rheb to GTP-Agarose beads, cells were harvested and suspended in lysis buffer. Lysates were centrifuged at 21,000 × *g*, 4 °C for 10 min, and supernatants were precleared with agarose beads for 1 h, rotating at 4 °C, followed by centrifugation at 3,000 × *g*, 4 °C for 5 min to separate the lysate from beads. Subsequently, equal amounts of protein were incubated with 30 µl of GTP-agarose (Sigma-Aldrich, G9768) with rotation for 2 h at 4 °C. GTP-agarose beads were washed three times in lysis buffer and eluted with 20 µl of sample buffer and boiling for 10 minutes at 70°C, resolved by SDS-PAGE, and proteins are analyzed via Western blot analysis

Generation of MAP4K3 and TFEB knockout cells using CRISPR/Cas9 genome editing

The 20 nucleotide guide sequences targeting human MAP4K3 were designed using the CRISPR design tool at http://crispr.mit.edu/ (Hsu et al., 2013) and cloned into a bicistronic expression vector (pX330) containing human codon–optimized Cas9 and the RNA components (Addgene).

The guide sequence targeting Exon 1 of human MAP4K3 is shown below.

MAP4K3: 5’ – TACCTTGTAGACGTCGCCGT – 3’

The single guide RNAs (sgRNAs) in the pX330 vector (1 μg) were mixed with EGFP (0.1 μg; Clonetech) and co-transfected into HEK293A cells using Lipofectamine 2000 (Life Technologies) according to manufacturer’s instructions. 24 hrs post transfection, the cells were trypsinized, washed with PBS, and re-suspended in fluorescence-activated cell sorting (FACs) buffer (PBS, 5 mM EDTA, 2% FBS and Pen/Strep). GFP positive cells were single cell sorted by FACs (UCSD; Human Embryonic Stem Cell Core, BDInflux) into 96- well plate format into DMEM containing 20% FBS and 50 μg ml-1 penicillin/streptomycin. Single clones were expanded, and screened for MAP4K3 by protein immunoblotting. Genomic DNA (gDNA) was purified from clones using the DNeasy Blood & Tissue Kit (QIAGEN), and the region surrounding the protospacer adjacent motif (PAM) was amplified with Phusion® High-Fidelity DNA Polymerase (New England Biolabs) using the following primers:

MAP4K3:

Forward: 5’ – GGAGCCGGGTGATTGTGA – 3’

Reverse: 5’ – AGAAGGGAGGTGGCAAAAAT – 3’

PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN) and cloned using the TOPO® TA Cloning (Life Technologies). To determine the exact mutations of individual alleles, at least 10 bacterial colonies were expanded and the plasmid DNA purified and sequenced.

Statistical Analysis

All data were prepared for analysis with standard spread sheet software (Microsoft Excel). Statistical analysis was done using Microsoft Excel, Prism 5.0 (Graph Pad), or the VassarStats website (http://faculty.vassar.edu/lowry/VassarStats.html). For ANOVA, if statistical significance ($p < 0.05$) was achieved, we performed post hoc analysis to account for multiple comparisons. The level of significance (alpha) was always set at 0.05.

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