A Review of Rheb Activation of mTORC1 and the Great Mystery of One Missing GEF

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Abstract

The mTORC1 pathway is involved in the regulation of cell growth and translation. The pathway has a complex web of activators and inhibitors to activate mTORC1. mTORC1 is regulated via a small GTPase called Rheb, which interacts directly with mTORC1. This GTPase and its GTPase activating protein (GAP), TSC1/2, have been widely studied to understand how the variety of regulators of mTORC1 interact with these proteins. Despite this, the guanine nucleotide exchange factor (GEF) of Rheb has yet to be identified. This review broadly analyzes Rheb and mTORC1, their structures, regulations, and interactions, and explores the mystery of the missing GEF for Rheb.

A Review of Rheb Activation of mTORC1 and the Great Mystery of One Missing GEF

Cells must adapt to the various environments that they encounter, whether it be the need to divide, the presence of a new metabolite, or the infection of a virus. Cells are capable of this adaptation through translating proteins to effectively react, but protein translation is an energetically expensive process. Therefore, the process of building a new protein through translation is highly regulated. A complex of proteins must form at the mRNA cap to allow the initiation of translation to begin; specifically, eukaryotic initiation factor 4E (eIF4E) must be bound to the mRNA cap *(1)*. However, this step is closely regulated by eIF4E-BP1, which binds to eIF4E and prevents its association with the 5' cap, thereby preventing mRNA loading onto the ribosome*(1)*. The mechanism to phosphorylate eIF4E-BP1 to remove it from eIF4E was discovered indirectly through the discovery of a chemical called Rapamycin *(2,3)*. *Streptomyces hygroscopicus* produces Rapamycin to defend against any fungal threats *(4)*; however, Rapamycin was found to be uniquely useful in studying yeast due to how it inhibits cell proliferation *(3)*. In yeast, *TOR1* and *TOR2* were shown to be the impacted genes whose products were inhibited, hence the name "Target of Rapamycin"; the homologues of these genes in mammals produce a protein named mTOR (mammalian target of Rapamycin) *(3)*. Later, mTOR was renamed to mechanistic target of Rapamycin *(2)*, highlighting that TOR also functions in non-mammalian cells like yeast, as referenced above. mTOR exists in two distinct complexes: mTORC1 and mTORC2, which differ by the associating proteins that bind to mTOR *(5)*. The mTOR complexes have a wide range of impacts in human cells, including proliferation and protein synthesis *(6)*. Whereas mTORC2 is involved in cell survival and cytoskeleton organization, mTORC1 activates proteins that lead to translation, cell growth, and more, and mTORC1 is the protein complex that phosphorylates eIF4E-BP1 in order to initiate protein

translation *(2,6)*. mTORC1 remains the more studied complex of the two due to the critical role it plays in many cellular pathways and the diseases that arise from a dysfunctional mTORC1 *(7)*. Separately, a protein was discovered in rat brains in 1994 which was very similar to a Ras protein. This protein was able to bind to GTP and had GTPase activities, but it was distinct from Ras proteins because it had a unique structure; this protein was also discovered to have been exceptionally transcribed in the hippocampus of rats, thus it was named Rheb (Ras homologue enriched in brain) *(8)*. The first human homologue of Rheb was quickly discovered in 1996 using a human fibroblast cDNA library *(9)*. It was later discovered that humans have two Rheb proteins, while many other organisms only have one *(10)*. Through trials and experiments, both Rheb proteins were found to be a keystone activator for the mTORC1 complex; they simply differ in where they are expressed in human tissues, Rheb1 in muscles mainly and Rheb2 predominantly in the brain *(10)*. Being a GTPase, Rheb must have a GTPase activating protein (GAP) to encourage it to move to its GDP-bound state, and it must have a guanine nucleotide exchange factor (GEF) to encourage it to discard GDP and rebind GTP *(11)*; these two regulators are crucial to understanding the function of Rheb and its regulation in the cell.

Overview of the Structure of Rheb

Rheb proteins are found across the Eukarya domain (with plants being the noteworthy exception) and is consistent in structure across its many variants *(12)*. The human homologue of Rheb, human Rheb (which shall be referred to simply as Rheb for the remainder of this review), is a GTPase in the Ras superfamily that has been discovered to reside on band q36 of chromosome 7 in the human genome *(9)*. This Rheb is 98.9% similar to the rat Rheb gene originally discovered *(9)*. The protein produced from the expression of this gene is approximately 21 kDa large and is a monomeric protein *(12)*. Rheb is 184 amino acids long; the first 169 amino acids act as the

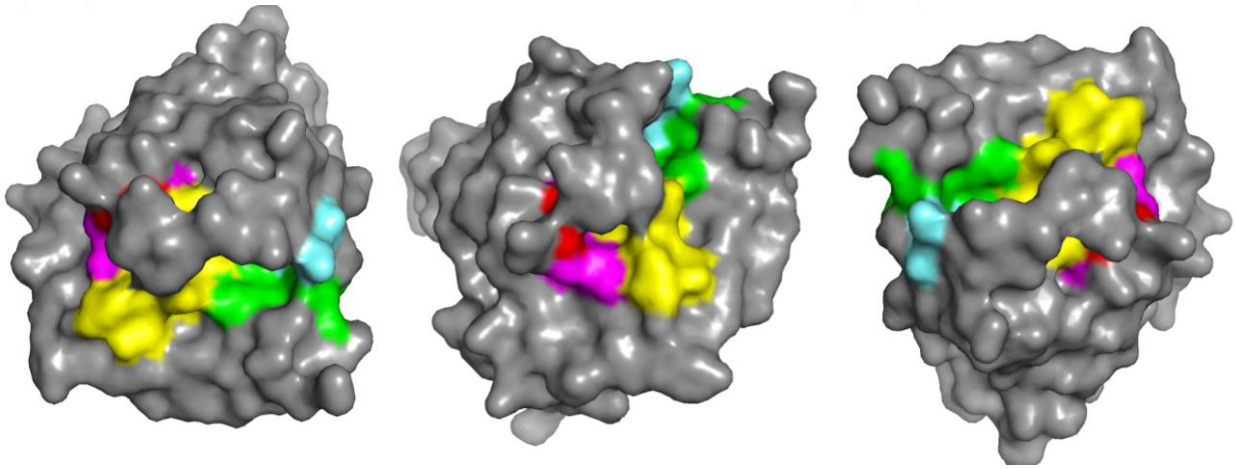
GTPase activity and the last 15 contain the CAAX motif *(12)*. This CAAX, which is -CSVM or -CHLM in humans *(13)*, motif is crucial for post translational modifications that occur to Rheb, which are critical for Rheb to bind to endomembranes within the cell *(12)*. The CAAX motif is first, and most importantly, farnesylated by farnesyltransferase at the cysteine of the CAAX motif, which is a lipid modification necessary for Rheb to be localized with mTORC1 which activates it *(13)*; the three C-terminal amino acids are cleaved during this process *(12)*. Despite it being well known that Rheb can be carboxylmethylated afterwards *(12)*, it is shown that this modification is not necessary for Rheb to activate mTORC1 *(13)*. Rheb contains six beta pleated sheets and five alpha helices, with two switch regions in two of the looping segments, which are uniquely consistent in structure despite GTP or GDP being bound *(13)*. These switch regions are of upmost importance for Rheb to interact with mTOR or any of the associating proteins in mTORC1 *(14)*. The hydrolytic activity of Rheb comes from five amino acid chains named "G boxes" *(15)*. These G boxes are highly conserved across species and are crucial to the ability of Rheb to bind and interact with the guanine ring of GTP *(15)*. Figure 1 shows the location of these G boxes visualized.

Figures 2 and 3 describe and show Rheb, both GTP and GDP bound, with different portions colored to differentiate. The images of the proteins clearly indicate major conformation change in Rheb. The most obvious change being that Rheb no longer wraps around the substrate but loosely holds it in the GDP-bound state. In general, Rheb has more pockets and bends around the substrate when bound to GTP than when bound to GDP. Regarding the residues noted for their proximity to the substrate, it is interesting that Rheb has two more residues in contact with GTP than GDP when either is bound to the protein. Each shares the same C-terminal residues interacting, namely Asn-119, Lys-120, Asp-122, Leu-123, Ser-149, Ala-150, and Lys-151. At

the N-terminal side of the sequence, each has the following chain interacting: Ser-16, Gly-18, Lys-19, Ser-20, Ser-21, Phe-31, Val-32, Asp-33, Ser-34, Tyr-35, Asp-36, Pro-37; however, the GTP bound conformation of Rheb also binds Arg-15, Val-17, and Thr-38. The greatest degree of difference comes in the small, unlikely chain that loops around to briefly bind to the substrate; for GTP-bound Rheb, this chain is Ala-62 and Gly-63, but, for GDP-bound Rheb, the chain of residues is Asp-60, Thr-61, and Ala-62. Lastly, the substrate is bounded to a magnesium ion cofactor in the protein. For GTP-bound Rheb, there are three residues surrounding the ion that may interact with it: Ser-20, Asp-36, and Thr-38. For GDP-bound Rheb, there are four residues surrounding the ion that may interact with it: Ser-20, Tyr-35, Asp-36, and Asp-60. Overall, these images and sequences reveal that Rheb undergoes major conformation changes when bound to GDP compared to GTP. These changes seem to be heightened in the middle portions of the amino acid chain. Additionally, the Rheb protein models for GTP-bound and GDP-bound were compared specifically for the region where the greatest structural change occurred. Figure 5 visualizes that conformational change due to the substrate binding. The amino acid chain that was discovered to be the most altered was as follows: Asp-33, Ser-34, Tyr-35, Asp-36, and Pro-37. In GDP-bound Rheb, the GDP was found in a depressed region, of which the amino acids 33-37 were found. In GTP-bound Rheb, the wall of this depression has conformed to bridge over the GTP as seen in Figure 4. Residues 33-37 bridge atop the GTP while residues 16-21 wrap around the GTP from below to form the domain where the GTP is contained.

Figure 1

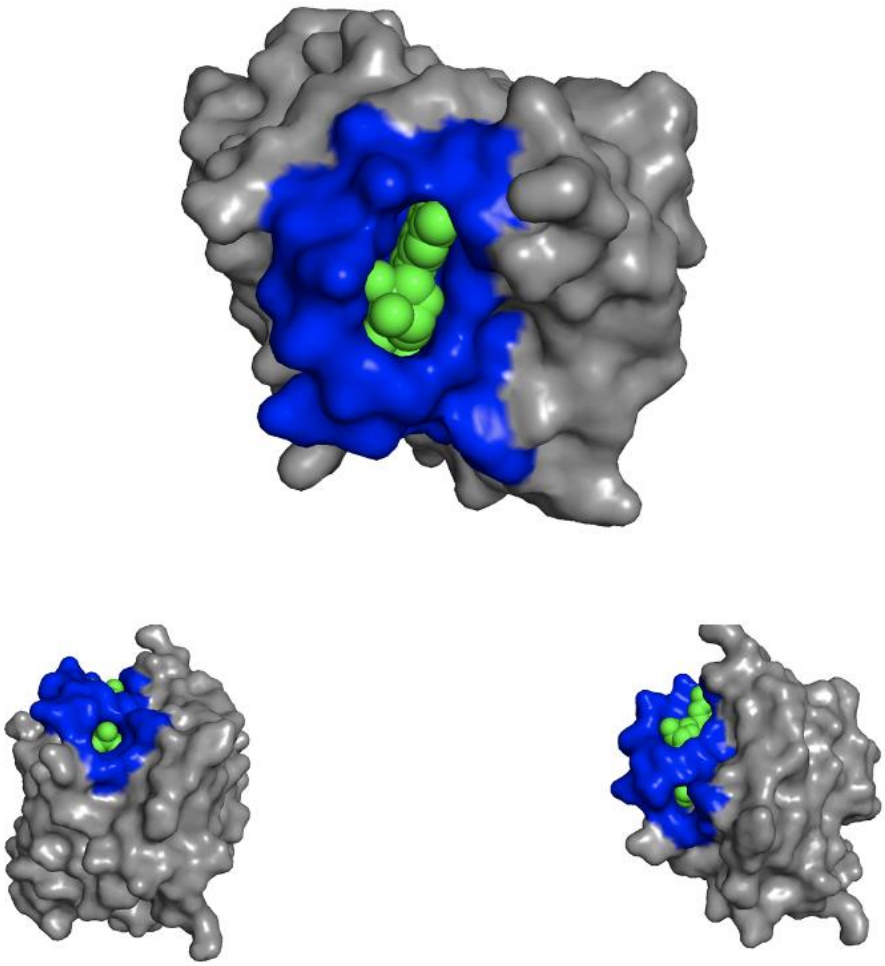
Colored Visualization of the G Boxes of Rheb for GTP Hydrolysis



The structure of Rheb with GTP was found on a PDB databank (PDB number: 1XTS *(16)*). The file was uploaded to Pymol and visualized. The five G boxes of the hydrolytic site of Rheb were colored as follows: the G1 box (Gly-13, Tyr-14, Arg-15, Ser-16, Val-17, Gly-18, Lys-19, and Ser-20) was colored yellow; the G2 box (Thr-38) was colored red; the G3 box (Asp-60, Thr-61, Ala-62, and Gly-63) was colored magenta; the G4 box (Asn-119, Lys-120, Lys-121, and Asp-122) was colored green; and the G5 box (Ser-149, Ala-150, and Lys-151) was colored cyan. The three views are different rotations of the protein to ensure a holistic visualization of all five G boxes.

Figure 2

Structure of Rheb Bound to GTP

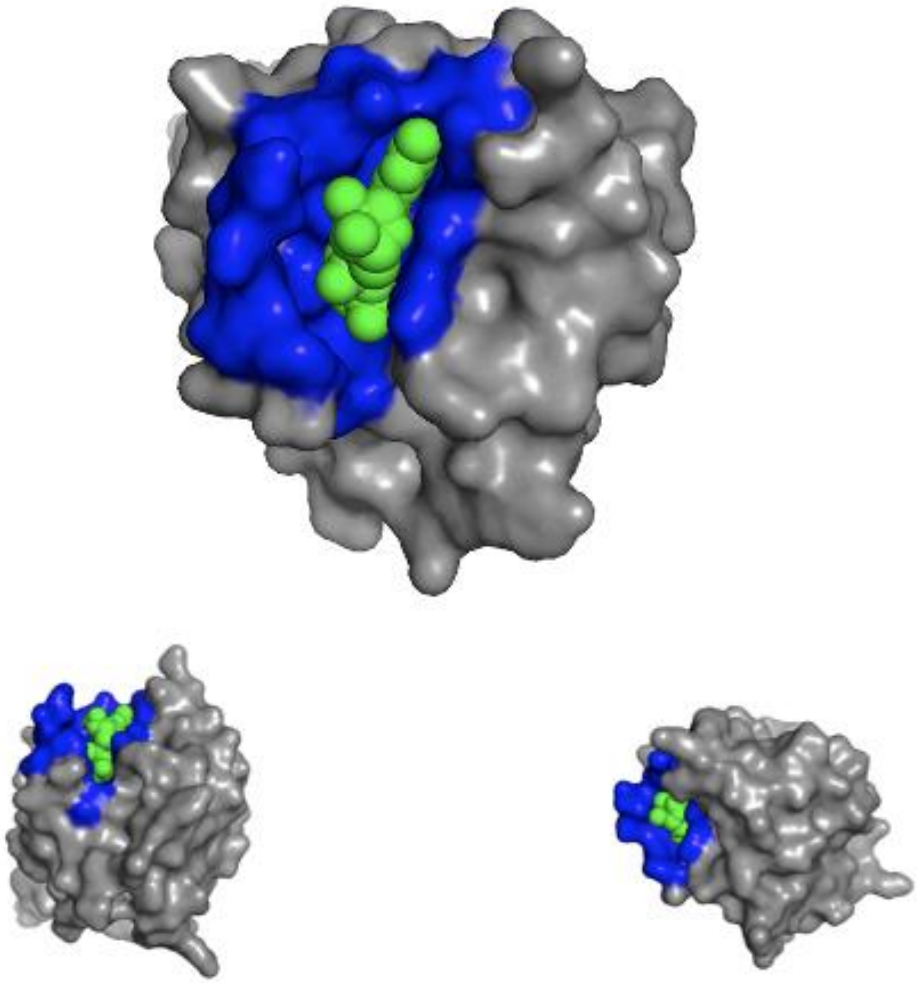


The structure of Rheb with GTP was found on a PDB databank (PDB number: 1XTS *(16)*). The file was uploaded to Pymol and visualized. Top: Best view of the GTP binding site. Left: Top view rotated 90 degrees downward. Right: Top view rotated 90 degrees left. The GTP was colored green and drawn as spheres, each amino acid that contacted the GTP was colored blue and was drawn as a surface model, and the rest of the protein was colored gray and drawn as a

surface model. The amino acids that come into contact with GTP are as follows: Arg-15, Ser-16, Val-17, Gly-18, Lys-19, Ser-20, Ser-21, Phe-31, Val-32, Asp-33, Ser-34, Tyr-35, Asp-36, Pro-37, Thr-38, Ala-62, Gly-63, Asn-119, Lys-120, Asp-122, Leu-123, Ser-149, Ala-150, and Lys-151. It is worth noting, the GTP is bound to a Magnesium ion, which is bound around Ser-20 and/or Asp-36 and/or Thr-38.

Figure 3

Structure of Rheb Bound to GDP

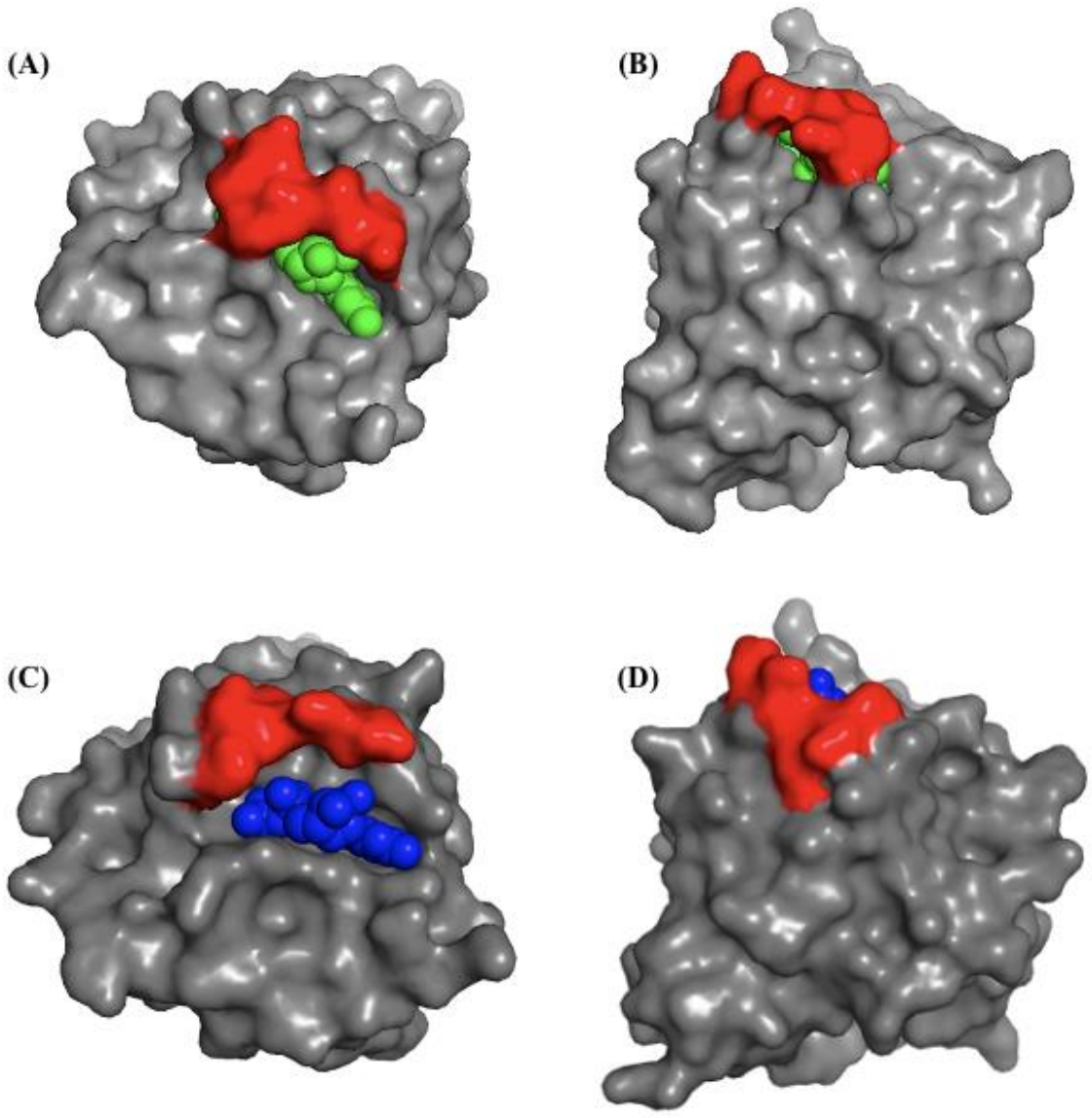


The structure of Rheb with GDP was found on a PDB databank (PDB number: 1XTQ *(16)*). The file was uploaded to Pymol and visualized. Top: Best view of the GDP binding site. Left: Top view rotated 90 degrees downward. Right: Top view rotated 90 degrees left. The GDP was colored green and drawn as spheres, each amino acid that contacted the GDP was colored blue and was drawn as a surface model, and the rest of the protein was colored gray and drawn as a surface model. The amino acids that come into contact with GDP are as follows: Ser-16, Gly-18,

Lys-19, Ser-20, Ser-21, Phe-31, Val-32, Asp-33, Ser-34, Tyr-35, Asp-36, Pro-37, Asp-60, Thr-61, Ala-62, Asn-119, Lys-120, Asp-122, Leu-123, Ser-149, Ala-150, and Lys-151. The cofactor for this protein, a Magnesium ion, is bound to the GDP alongside the following residues: Ser-20 and/or Tyr-35 and/or Asp-36 and/or Asp-60.

Figure 4

Structures of GTP-Bound Rheb and GDP-Bound Rheb Compared with Emphasis of Key Structural Change



The structure of Rheb with GTP was found on a PDB databank (PDB number: 1XTS *(16)*) and the structure of Rheb with GDP was found on a PDB databank (PDB number: 1XTQ *(16)*). The

files were uploaded to Pymol and visualized. After comparison, the sight of greatest change was highlighted red; this sight contained the following residues: Asp-33, Ser-34, Tyr-35, Asp-36, and Pro-37. In all images, GTP was colored green, GDP was colored blue, the region of interest was colored red, and the rest of the protein was colored gray. (A) Image of GTP-bound Rheb from above the binding site. (B) Image of GTP-bound Rheb rotated to visualize the protein surface with gaps due to the bridge conformational change. (C) Image of GDP-bound Rheb from above the binding site. (D) Image of GDP-bound Rheb rotated to visualize the "wall" of the depressed binding site region formed by the region of interest.

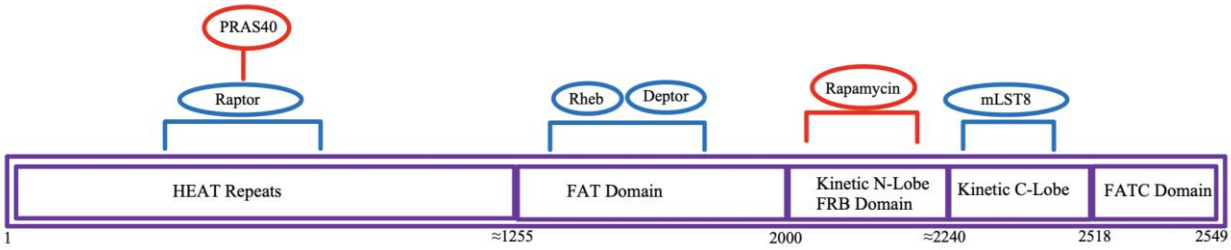
mTORC1 Structure and Functions Briefly

The *mTOR* gene produces a 2549 amino acid polypeptide chain *(17)*. This protein, mTOR, is the foundational subunit for all phosphorylation that occurs due to the mTORC1 and mTORC2 complexes *(7)*. mTOR consists of repeating HEAT domains, a FAT domain, FATC, FRB, and two kinetic domains *(3)*. The FAT domain is a looping section of 28 alpha helices that clamps the kinetic domain, and the FATC domain is critical to hold the kinetic domain in its correct place *(18)*. The HEAT domain is a series of solenoids that allow for binding to other domains in the complex, like the FAT domain and domains in RAPTOR *(19)*. The FRB domain binds into the N-terminal lobe (one of the two lobes of the kinetic domain), and the lobes of the kinetic domain create a cleft between them where binding occurs *(18)*. Both complexes obviously contain mTOR but also bind Deptor, mLST8, and PRAS40 for regulation *(7)*; it is worth noting that many do not regard PRAS40 as a subunit of the complex since it binds to RAPTOR (regulatory-associated protein of TOR) to cause inhibition *(19)*. As seen in Figure 5, Deptor binds to mTORC1 at the FAT domain of mTORC1 *(20)*, whereas mLST8 binds to mTORC1 at the C-terminal lobe of the kinetic domain *(18)*. Lastly, RAPTOR binds to the HEAT repeats in

mTORC1 *(3)*. The crucial difference in the structure of the complexes is that mTORC1 binds to RAPTOR, whereas mTORC2 binds to rictor (rapamycin-insensitive companion of TOR) *(5)*. mTORC2 phosphorylates AKT, SGK1, and PKC which are all serine/threonine kinases *(18)* to affect cell survival and the cytoskeleton *(6)*. Broadly speaking, mTORC1 controls many aspects of cellular stability, like lipogenesis, protein synthesis, and autophagy *(19)*, but, more specifically, mTORC1 phosphorylates eukaryotic initiation factor 4E binding protein 1 (eIF4E-BP1) at The-37 and Thr-46 *(6)*, and it activates ribosomal protein S6 kinase (S6K) *(5)*, which leads directly to increased translation and, later, cell growth *(3)*.

Figure 5

Structure of mTOR and Locations of where Regulatory Proteins of mTORC1 Bind



The translated product of mTOR has the following six domains: HEAT repeats (1 to 1255 *(21)*), a FAT domain (residues 1255-2000 *(18)*) , N-terminal lobe of the kinase domain (residues 2000-2240 *(18)*), the FRB domain (which is embedded in the N- terminal lobe), the C-terminal lobe of the kinase domain (residues 2240 to 2518 *(18)*), and the FATC domain (residues 2518-2549 *(18)*) *(3)*. For mTORC1 but not mTORC2, raptor binds to the heat domains *(3)*. Rheb and Deptor each bind within the FAT domain, but they are not present simultaneously, since Deptor is released prior to activation by Rheb *(20)*. Rapamycin binds to the FRB domain after forming a

complex with another protein *(3)*. Lastly, mLST8 binds at the C-terminal lobe of the kinase domain *(18)*.

Regulation of mTORC1

The activity of mTORC1 is regulated by amino acids, glucose, oxygen *(19)*, stress, and growth factors *(3)*, and is summarized in Figure 6. Activation of mTORC1 at large causes RAPTOR to bring the complex to the lysosome and other endosomes *(18)*. In contrast, mTORC2 is only directed by growth factors that signal cell survival *(3)*.

The most important and direct regulator of mTORC1 is the monomeric GTPase Rheb, which activates mTORC1 when in its GTP bound state *(5)*. Many studies have gone into other possible GTPases and proteins that may activate mTORC1, like Ral, Cdc42, or K-Ras, but mTORC1 has been shown to be Rheb specific *(10)*. While mTORC1 is exclusive to Rheb, Rheb is also relatively exclusive to mTORC1, for a GTP bound Rheb has been shown to have no effect on mTORC2 *(10)*. Many regulators of Rheb have been discovered, but none are as important as TSC1/2, its most prominent inhibitor *(7)*. TSC2 possesses GAP activity towards Rheb that is only activated when it exists in complex with TSC1, forming TSC1/2. In this state it causes Rheb to hydrolyze GTP and no longer be active in the mTORC1 pathway *(5)*. Indeed, TSC1/2 is a central regulator of mTORC1 activity, and many proteins either activate or inhibit mTORC1 based on their effect on TSC1/2. Interestingly, mTORC2 phosphorylates and activates the serine/threonine kinase AKT, a known inhibitor of TSC1/2 function *(22)*; therefore, mTORC2 is an activator of mTORC1 via AKT *(3)*. Increased insulin levels lead to the activation of PI3K which phosphorylates phospatidylinositols at the plasma membrane to form docking sites for AKT, allowing AKT to be activated *(23,24)*, thus mTORC1 is activated by insulin via PI3K. Another pathway includes the extracellular signal-regulated kinase complex (ERK1/2), a

serine/threonine kinase that phosphorylates those residues when adjacent to a proline *(25)*. ERK1/2 inhibits TSC1/2 via phosphorylation which leads to mTORC1 activation *(5)*, but ERK1/2 also activates p90 ribosomal 6S kinase (RSK) using the same mechanism *(25)*, and RSK inhibits TSC1/2 by phosphorylating Ser-1798 to have the same effect as ERK1/2 *(22)*. Mitogen activated protein kinase kinase (MEK) activates mTORC1 by activating ERK1/2 by phosphorylating a threonine and tyrosine on ERK1/2 *(25)*. MEK is activated by Raf when Raf phosphorylates a serine on MEK *(25)*; Raf is then activated by a Ras GTPase *(26)*. This activation occurs when Ras causes a Raf kinase to activate and phosphorylate Raf in an unknown location *(25)*. If a cell has an excess of AMP, meaning that there is a low concentration of ATP, then AMP activated protein kinase (AMPK) will be activated, and that kinase phosphorylates TSC1/2 to keep TSC1/2 united as a complex *(23)* which would allow TSC1/2 to continue inhibiting mTORC1; AMPK specifically phosphorylates TSC2, at Ser-1345 *(27)*. In hypoxic conditions, REDD1 is activated to inhibit mTORC1 *(28)* by indirectly encouraging TSC2 to bind to TSC1 through an unknown manner *(29)*.

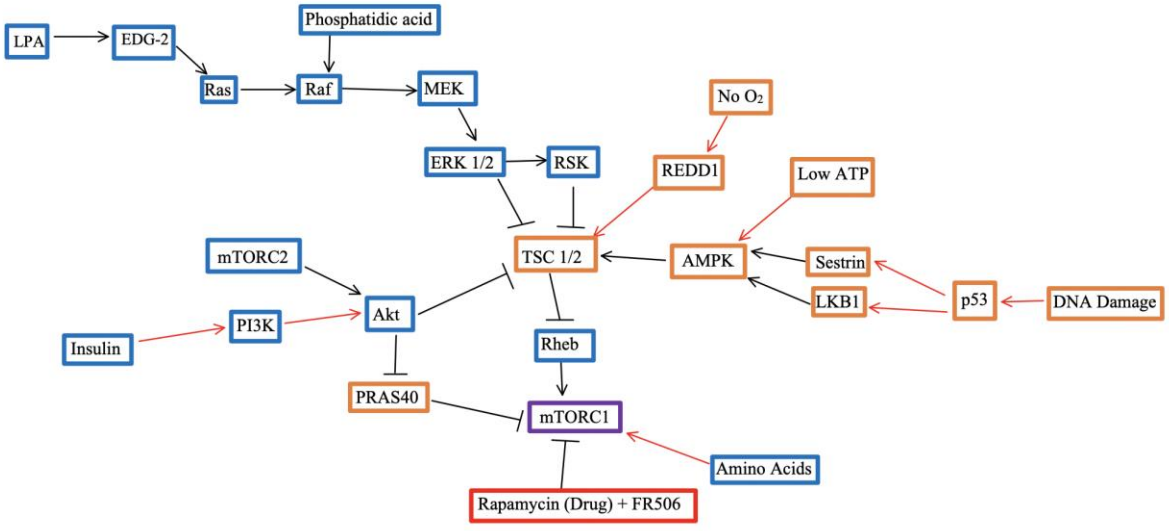
p53, a protein produced to halt the cell cycle if DNA damage occurs, promotes the transcription of Sestrin and LKB1 to inhibit mTORC1 and discourage cell proliferation *(28)*. Sestrin and LKB1 each inhibit mTORC1 by phosphorylating Thr-172 on AMPK to activate AMPK to phosphorylate TSC1/2 to inhibit mTORC1 *(28,30)*. One last TSC1/2 pathway is through phosphatidic acid, which has two forms. Extracellular phosphatidic acid is converted to Lysophosphatidic acid (LPA) by phospholipase A; LPA binds to the EDG-2 receptor on the plasma membrane, which triggers the Ras GTPase pathway and ERK1/2 activity *(5)*. That same receptor, EDG-2, leads to an increase in phospholipase catalysis, which causes

phosphatidylcholine to break apart to phosphatidic acid and choline; the intracellular phosphatidic acid then activates Raf *(5)*.

Pathways that do not include TSC1/2 are also abundant in mTORC1 regulation. Rapamycin, the namesake compound for mTOR, can bind to FR506 binding protein, which then together bind to mTOR to deactivate it by binding to the FRB domain *(3)*. Amino acids are also likely candidates for bypassing TSC1/2 and directly controlling mTORC1 *(23)*; specifically, leucine is known to activate mTORC1 *(5)*. These amino acids activate a series of regulators called Rag proteins to bring mTORC1 to the lysosomal surface *(3)*. Similar to amino acids, nutrients have been shown to bring mTORC1, via RAPTOR, to the lysosomal surface, which leads to its activation *(19)*. PRAS40 has been discovered to bind to RAPTOR and completely inhibit the kinase activity of mTOR *(31)*. Lastly, AKT, activated by insulin, has been shown to phosphorylate and inhibit PRAS40, thereby, activating mTORC1 *(31)*.

Figure 6

mTORC1 Signaling Pathway



All the inhibitors and activators of mTORC1 that are discussed are shown. Black arrows are used for interactions that are directly related, whether it be a phosphorylation, binding, or receptor releasing the G protein. Red arrows are used for relationships that have are indirect and have other steps. The color Purple is used for mTORC1. Orange is used for regulators that lead to the inhibition of mTORC1. Blue is for any regulators that ultimately activate mTORC1. Red is used for Rapamycin since it is an inhibitor that is originally foreign to the cell and must be introduced. As seen above, there are many pathways that regulate mTORC1. Most pathways function by activating TSC1/2 which inhibits Rheb from activating mTORC1 *(5)*. To begin, Ras, a G protein, activates Raf to phosphorylate MEK to phosphorylate ERK1/2 *(26)*. ERK1/2 both inhibits TSC1/2 and activates RSK to inhibit TSC1/2 *(5,22)*. Phosphatidic acid activates this pathway in two distinctly different mechanism (one intracellular and one extracellular) *(5)*. The other pathway that ultimately activates mTORC1 through TSC1/2 begins with extracellular insulin activating PI3K which activates Akt *(23)*. mTORC2 also activates Akt to inhibit TSC1/2 *(22)*. Akt is also known to inhibit PRAS40, a protein known to directly inhibits mTORC1 *(31)*. The last activator to be mentioned are amino acids, which are known to directly activate mTORC1 *(23)*. There are multiple pathways shown that activate TSC1/2 to inhibit mTORC1. First, hypoxic conditions activate REDD1, and REDD1 activates TSC1/2 *(28)*. Low ATP levels in the cell activates AMPK, and AMPK activates TSC1/2 *(23)*. DNA damage activates AMPK as well through the activation of Sestrin and LKB1 via p53 *(28)*. Lastly, Rapamycin, when added to a cell, will directly inhibit mTORC1 by binding to FR506 *(3)*.

Activation and Regulation of Rheb

Rheb, being the activator of mTORC1, is tightly regulated through multiple pathways. *(12).* Rheb naturally switches between its GTP bound and GDP bound states *(13)*, but, as with any GTPase, the GAP that activates the intrinsic GTPase activity of Rheb is necessary for normal cellular function, with the GAP being TSC2, as previously discussed. The inverse is also true; GTPases can naturally switch from GDP bound to GTP bound states on their own, but a GEF encourages the GTPase to release GDP and rebind to GTP *(13)*. Using mutation studies, it was shown that TSC1/2 binds to Rheb specifically at the tuberin (TSC2) subunit *(12)*. TSC1/2 binds using Arg-388, which was hypothesized to be a crucial amino acid in the GAP activity domain *(12)* and causes its GAP activity at Asn-1634 through a mechanism known as an "Asparagine thumb" *(12)*. Though the TSC1/2 complex is known to be the GAP for Rheb *(32)*, the GEF for Rheb remains unknown *(12)*. Interestingly, Rheb has GAP independent regulation; when a cell is running out of energy, a cascade travelling from p38β to PRAK through p38β kinase *(12)* phosphorylates Rheb at Ser-130, which causes Rheb to lose affinity for guanine molecules, thus inhibiting Rheb and inhibiting mTORC1 without using TSC1/2 *(12).* Another system that may be independent of TSC1/2 was shown when Rheb had less affinity to GTP when a cell was amino acid deprived *(23)*, but this may simply be a pathway that causes TSC1/2 activity to increase. Proper editing and targeting of Rheb allows for Rheb to activate the mTORC1 pathway. Rheb can be edited in many ways, including farnesylation, prenylation, and post-prenylation. Farnesylation of Rheb is well known to be crucial to Rheb being localized at the lysosomal membrane *(12)*. Farnesylation was further shown to be necessary when experiments using Farnesyltransferase Inhibitors caused mTORC1 activation to decrease greatly *(13)*. However, post-prenylation processes are debated on their usefulness; one review argued that they are

crucial for Rheb to be localized *(12)*, whereas another says post-prenylation modifications like carboxymethylation and proteolytic cleavages are not necessary for the activation of mTORC1 via Rheb *(13)*. Researchers also discovered that Rheb can be inhibited by small molecules, like NR1, that can bind to the switch domain of Rheb and inhibit any activity with mTORC1 *(7)*. Lastly, at the genetic level, Rheb has been shown to be regulated by microRNA-155 by binding to the 3' end of the *RHEB* gene *(12)*.

The Interaction Between Rheb and mTORC1

Correct localization is needed for Rheb and mTORC1 to interact. Though some articles argue that mTORC1 and Rheb mainly reside on the nuclear membrane-ER membrane system *(33)*, it is generally accepted that mTORC1 is localized by a RagA/RagC or RagB/RagD complex that directs mTORC1 to the lysosome *(12)*. Once in the vicinity, a complex formed by p18, p13, MP1, and other proteins guides the binding of the Rag complex onto the lysosome, and mTORC1 is pulled along with it *(12)*. In order for Rheb to be bound to the lysosome, it must first be farnesylated, but that does not immediately form a mTORC1/Rheb complex *(34)*. Rheb bound to the ER is insufficient to activate mTORC1, it must be on a vesicle from the Golgi body *(34)*. It is not nearly enough to be in a location to begin activity, for Rheb must not only be in its GTP bound state *(13)* but also have its effector domain functioning *(10,13)*. Studies have shown that the GTP bound state is necessary, despite Rheb proteins that are unable to bind to guanine molecules but are still capable of binding to mTOR *(35)*. This is due to the conformational change that results from the binding of Rheb; without a GTP, the change will not result in activation. Once every component is in place, Rheb and mTORC1 must interact. Many studies have gone into discovering which proteins of mTORC1 actually bind to Rheb. One study used FRET and very precise photon excitement to discover very specific binding of Rheb and the

mTOR protein itself *(33)*. Rheb was discovered to interact with mTOR at the amino terminal lobe of its catalytic domain *(33)*. This study showed that Rheb does not directly interact with RAPTOR *(33)*. Another study revealed that RAPTOR effects the binding and activity of Rheb with mTORC1, but Rheb and RAPTOR, again, were never directly bound to one another *(10)*. Rheb physically binds to mTORC1 via its Switch II region, which is a looping region of its secondary structure that connects to mTORC1 *(14)*. It was shown that this is not through the hypothesized binding of Rheb to FKBP38, since siRNA knockouts of FKBP38 had no effect on mTORC1 activation *(10)*. The binding of Rheb to mTORC1 activates the kinase activity of mTOR but not through the anticipated means of enhancing the catalytic domain of mTOR *(10)*, since mTOR autophosphorylation was never significantly induced by Rheb binding to mTOR. Rheb causes an allosteric shift in mTORC1, which causes the active sites to be in position for substrates to bind *(19)*. In detail, this occurs by Rheb causing the FAT regions of mTOR to realign and rotate, which leads to substrate binding *(19)*. This has been verified by other studies finding greater affinity between eIF4e-BP1 and mTORC1 after Rheb binds *(10)*. RAPTOR guides the substrate binding once the conformational change occurs, and the catalytic site phosphorylates the substrates, which are commonly eIF4e-BP1 or S6K *(13)*.

The Missing GEF

Every researcher who has studied mTORC1 and Rheb recognize that something is missing from the understanding of the entire pathway: the identity of the missing GEF *(13)*. The debate for what is possibly the GEF for Rheb has naturally drawn controversy from multiple laboratory teams. The only major proposition proposed is a protein named translationally controlled tumor protein (TCTP) *(36)*. TCTP was discovered years prior to the mTORC1 conversation, and its potential GEF activity was never analyzed, despite structurally similarities to other GEFs,

namely mammalian suppressor for sce4 (Mss4) *(37,38)*. Mss4 is a GEF for the Rab family of GTPases, which activate various functions in vesicle formation and movement *(38)*. The GEF activity of TCTP was first analyzed in fruit flies; *drosophila* TCTP (dTCTP), the fruit fly variant, was found to fit all the criteria necessary *(36)*. Research found that reducing dTCTP in a cell has the same effects as a cell mutated to not synthesize Rheb, like cell number reduction and cell size reduction *(36)*. dTCTP also displayed GEF activity with Rheb in fruit flies *(36)*. The human variant (hTCTP) was analyzed to be structurally very similar to dTCTP and have similar impacts within a cell *(36)*. Immediately, two papers resisted: one titled "Biochemical characterization of TCTP questions its function as a guanine nucleotide exchange factor for Rheb" and the other titled "Reevaluating the roles of proposed modulators of mammalian target of rapamycin complex 1 [mTORC1] signaling." However, one team analyzed all the prior studies and completed a thorough analysis of hTCTP to holistically answer this question *(37)*. Using in vitro and in vivo tests, the GEF activity of hTCTP was further supported *(37)*. This team hypothesized that a glutamine at position 12 was likely either the site containing the GEF activity or the binding site for Rheb, since it appears to be conserved among many TCTP variants across species *(37)*.

Going a different direction, a study done on *C. elegans* found that CGEF-1 expression led to mTORC1 activation and that CGEF-1 knockout led to similar results as a Rheb or Raptor knockout *(39)*. CGEF-1 was found to contain both a Dbl Homology and a Pleckstrin Homology, and their results implied that CGEF-1 was able to bind directly to Rheb in vivo *(39)*. This study also used showed that human Dbl binds to Rheb in human cells *(39)*.

The Discoveries of Other GEFs

Dbl GEFs

In studying oncogenes, one research team discovered an oncogene product that uniquely contained GEF activity *(40)*. This protein, called Dbl, was discovered to have GEF activity with specifically CDC42 and also other Rho-related GTPases *(41)*. In order to confirm the GEF activity of Dbl, the researchers used radioactive GDP for GDP dissociation assays and radioactive GTP for GTP uptake assays *(42)*. Dbl was found to have a domain that was crucial for its GEF activity, the DH domain (Dbl homology) *(43)*. Further analysis of the Rho family of GTPases revealed that most GEFs contain a PH domain (pleckstrin homology) which is C-terminal to the DH domain *(43)*. Whereas DH is thought to be the GEF activity domain, PH has been hypothesized to either help in targeting the GTPases in question or enhance the DH domains activity *(43)*. Using the Dbl homology, more GEFs have been isolated including Vav, Ect-2, and many others *(41)*. Almost every GEF in the Ras family of GTPases contain the DH and PH domains, but simply having one or both domains in some form present does not guarantee GEF activity *(41)*.

The Road to the Son of Sevenless

Ras proteins are a large family of small G proteins that cover many cellular pathways *(44)*. Though known for their interactions with receptor tyrosine kinases (RTKs), it was not until 1991 that the first GEF for a Ras protein was confirmed biochemically *(45)*. In 1987, two separate teams discovered CDC25 and observed how mutations in the CDC25 gene causes RAS1 and RAS2 normal functionality to decrease *(46,47)*. Then, in 1991, a team used yeast cultures to test the hypothesis that CDC25 has GEF activity with Ras *(44)*. This team used two separate assays after culturing yeast with normal functioning Ras proteins; first, they used tritiated GDP to

measure the rate of GDP loss across the concentrations of CDC25, then they used ^{32}P labeled GTP to measure GTP uptake in response to various concentrations of CDC25 *(44)*. These results, along with other methods to ensure controlled measurement apart from the other sources of guanine nucleotides and error that could occur in a cell, pointed to a joint GDP removal and GTP binding action induced by CDC25 *(44)*. Separately, in 1990, ste6 was found to have the same impacts on RAS1 and RAS2 as CDC25 and was later assumed to act as a GEF as well *(45)*. After analyzing the similarity between these two GEFs, a similar sequence of residues was discovered, which was later named as the exchange factor homology domain *(45)*, which is distinct from the Dbl Homology *(41)*. Using this homology domain, researchers discovered the mammalian homologue to CDC25, named RasGRF *(45)*. A team in 1992 confirmed the GEF activity of RasGRF using tritiated GDP and ^{32}P labeled GTP separately to measure the dissociation rates when a variety of concentrations of RasGRF was added *(48)*. In 1991, a separate team was researching the RTK sevenless pathway in the fruit fly and discovered a protein they called the Son of Sevenless (SOS) *(49)*. This protein was homologous to CDC25 as well, having the exchange factor homology domain *(49)*. Two mammalian homologues to SOS were discovered later *(45)*. The activity of the human homologue of SOS (hSOS1) was studied first by knocking out CDC25 in yeast and using a fragment of the hSOS1 gene containing the Exchange Factor Homology Domain and then studying the interactions of hSOS1 with growth factors *(50)*. The first analysis showed that hSOS1 contains GEF activity, and the second analysis revealed that hSOS1 also has a proline rich region, allowing it to bind to SH3 domains *(50)*. SH3 domains are commonly coupled to SH2 domains *(45)*; RTKs and the proteins that RTKs phosphorylate bind to one another via SH2 domains, and then those proteins may have a SH3 domain to then continue the signaling pathway *(45)*.

β-Pix

The PAK family of kinases has been discovered to regulate gene expression, cytoskeletal arrangement, and apoptosis *(51)*. These kinases are regulated by Rac and CDC42 GTPases *(51)*. In 1998, a team studying the relationship between the PAK family of kinases and Rac and CDC42 GTPase discovered the GEF for Rac1 *(52)*. Analysis of this protein yielded discovery that it contains a Dbl homology, Pleckstrin homology, and SH3 domain *(52)*. In order to confirm their discovery, the team used a known GEF for CDC42 and Rac1, ABR, then compared their discovery to this one *(52)*. Their discovered GEF had a higher uptake of tritiated GDP and a greater dissociation of ^{32}P labeled GDP *(52)*. The SH3 domain of this protein, named PIX, was discovered to be highly selective to only PAK proteins, which implied it may only function with CDC42 and Rac1, not more broadly like other GEFs *(52)*.

G-Protein Coupled Receptors

To broaden the scope, G-Protein Coupled Receptors (GPCRs) are transmembrane proteins that act as signal receptors to transduce the signal into the cell *(53)*. GPCRs are distinct for having seven transmembrane helices and being stimulated by an extracellular ligand to activate an effector G-Protein intracellularly *(54)*. GPCRs, though not named that initially, were first discovered 1980, when cellular receptors were doubted to exist *(55)*. Their receptor functionality was discovered first, where it could bind to a ligand then lead to the activation of adenylyl cyclase *(55)*. A noteworthy difference in affinity to specific ligands then led to more experimentation *(55)*; upon adding GTP and nucleotides into the system, it was discovered that affinity distinctly changed, where guanine led to a lower affinity state of the receptor *(56)*. This led to the ternary complex model for the receptor, where the receptor would use a nucleotide to lead to an active state of the protein associated with it intracellularly *(56)*. Though distinctly

different and somewhat indirect than how other GEFs were discovered, the team discovered this GEF activity, though not even regarded as such at the time, by analyzing the effect of GTP and guanine on the efficiency of activation of adenylyl cyclase *(56)*. Separately, in 1995, a series of studies on the molecular mechanism of adenylyl cyclase inhibition led to the discovery of heterotrimeric G proteins *(57)*. Heterotrimeric G proteins are GTPases, like Rheb or Ras, but are unique in that they have three subunits, while the Ras superfamily has only one subunit *(58)*. This discovery connected the receptor studies to G proteins and allowed for the conclusion that GPCRs were the GEFs for heterotrimeric G Proteins *(57)*.

Finding a GEF

The discovery of every prior GEF discussed came in a similar manner, not by testing each protein with different ones that may have a homology somewhere but analyzing the GTP uptake and GDP dissociation. The analyses for TCTP as the GEF for Rheb lacks this. The first paper to propose TCTP as the GEF for Rheb made their claim based on similar downstream effects that TCTP was shown to have *(36)*. The team that attempted to confirm those results did so by analyzing the crystal structures looking for a homology between the Rheb and TCTP and using a GDP dissociation assay *(37)*. Though TCTP may be the GEF for Rheb, more traditional analyses need to be done. TCTP lacks a genetic analysis that shows the crucial Dbl Homology and Pleckstrin Homology, and no GTP uptake assays have been completed. On the other hand, CGEF-1 has a complete genetic analysis that shows a Dbl Homology and Pleckstrin Homology *(39)*. However, as promising as binding results seem for CGEF-1, assays measuring GDP dissociation and GTP uptake are needed to conclusively regard CGEF-1 as the GEF to Rheb. Lastly, both hypotheses posit their proteins based on results from knockout experiments that have similar downstream impacts in the cell overall; however, these results could come from a

variety of aspects of the mTORC1 pathway, since there are many branching pathways related to mTORC1, and there are multiple G proteins involved in mTORC1 regulation.

Conclusion

Many mysteries regarding Rheb still exist. It is also crucial to discover the GDP dissociation inhibitor for Rheb (GDI) *(12)*. The GDI is the direct opposite of the GEF; whereas the GEF encourages the replacement of GDP with GTP, the GDI inhibits the GTPase from releasing GDP *(12,59,60)*. This occurs by the GDI binding to the GTPase *(59,60)*; therefore, it is necessary for the GDI to be removed before the GEF can have an impact *(60)*. Further in vitro studies are also required to fully understand the mechanism of Rheb binding and activating mTOR and how RAPTOR is involved *(13)*. But, without a doubt, the mystery of the missing GEF remains the greatest unanswered question for Rheb *(13)*. Though TCTP has been proposed, there is a lack of conclusive evidence and a holistic mechanism understood to truly accept it *(13)*; CGEF-1, as well, appears promising, but further studies are required to inscribe CGEF-1 as the GEF to ground future mTORC1 regulation research.

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