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Defining the biochemical functions of the TSC complex and its regulation of Rheb-mTORC1 signaling

A dissertation presented

by

Sophie Evarts Lockwood

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

Harvard University

Cambridge, Massachusetts

May 2023

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Dissertation Advisor: Professor Brendan D. Manning Sophie Evarts Lockwood

Defining the biochemical functions of the TSC Complex and its regulation of Rheb-mTORC1 signaling

ABSTRACT

Nutrients and growth factors are sensed by a ubiquitous signaling network that converges on the protein kinase complex mechanistic target of rapamycin (mTOR) complex 1 (mTORC1). mTORC1 functions to integrate a diverse set of growth cues to coordinate the important anabolic processes fundamental to cell growth and proliferation. Upstream of mTORC1, growth factor signaling pathways impinge on the tuberous sclerosis complex (TSC) complex, comprised of the tumor suppressors TSC1 and TSC2, and a third component, TBC1D7, to inhibit its ability to regulate the direct mTORC1 activator, Rheb. The pathways that impinge on the TSC complex are comprised of some of the most common oncogenes and tumor suppressors genetically altered in human cancers. Moreover, direct mutations on TSC1 and TSC2 lead to the genetic tumor syndrome tuberous sclerosis complex (TSC) and have also been identified in sporadic cancers. In all these settings, mTORC1 is aberrantly activated and results in uncontrolled cell growth.

The TSC complex acts as a GTPase Activating Protein (GAP) to stimulate GTP hydrolysis on Rheb, converting it from its active to inactive form, and this regulatory function is mapped exclusively to a small c-terminal domain on TSC2. Although significant progress has been made towards understanding the canonical GAP domain of TSC2, many longstanding questions underlying the mechanistic function of the TSC complex and its regulation of Rheb remain unanswered.

Through mechanistic studies, I have identified a novel binding interaction between the TSC complex and Rheb that occurs outside of the canonical GAP domain of TSC2. I find that the TSC complex preferentially engages Rheb in is inactive form and that this interaction occurs in a highly phosphorylated region of TSC2 containing the most essential growth factor-stimulated phosphorylation sites. Finally, I show that this interaction appears to be important for complete mTORC1 suppression upon growth factor withdrawal from the cell. Collectively, these studies have revealed previously unrecognized regulatory functions of the TSC complex and provide updated molecular details towards our understanding of the rheb-mTORC1 signaling axis in both normal settings and disease.

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CHAPTER 1:

Introduction

mTORC1: a major regulator of cell growth and proliferation

Overview

The growth and survival of all organisms is fundamentally dependent on the ability of an organism to adapt to changes in nutrient abundance. As such, cells must be able to sense and respond to growth signals, both within the cell and in its surrounding environment, to alter metabolic processes accordingly. The anabolic (use of nutrients and energy to synthesize macromolecules) and catabolic (breakdown of macromolecules to generate energy) processes underlying regulated growth are controlled by an extensive signaling network. This signaling network is highly connected through major signaling nodes that sense and integrate diverse signals to coordinate proper growth of the cell, tissue, and organism[1].

One of the major and essential nodes in this intricate network, is the serine/threonine protein kinase complex mechanistic target of rapamycin (mTOR) complex 1 (mTORC1)[2]. The catalytic subunit of mTORC1, mTOR, exists in a second protein complex called mTORC2. mTORC1 and mTORC2 are distinct in both their biochemical composition and regulatory role in the cell. In addition to the mTOR kinase, both complexes contain the protein mLST8. mTORC1 is defined by the additional subunit Raptor while Rictor, Protor and mSIN1 define mTORC2. While less studied than mTORC1, mTORC2 is believed to regulate cell survival, proliferation and actin cytoskeleton dynamics, primarily through activating or stabilizing phosphorylation events on members of the AGC protein kinase family, such as Akt[3].

In contrast, mTORC1 functions as a master signaling node regulating cell growth and metabolism. mTORC1 is acutely responsive to growth promoting signals such as exogenous growth factors, intracellular nutrients in the form of amino acids, glucose and oxygen, as well as energy status (ATP) and initiates a shift from catabolic to anabolic processes such as protein,

lipid, and nucleotide synthesis. Given that these anabolic processes demand abundant nutrients and energy, the activation state of mTORC1 must be tightly controlled to balance the supply of nutrients and energy with the demands for producing biomass to drive growth. Not surprisingly, aberrant activation of mTORC1 underlies the pathophysiology of numerous human diseases ranging from cancer to metabolic diseases, neurological disorders, and autoimmune and inflammatory diseases[4].

Downstream processes controlled by mTORC1

mTORC1 promotes protein synthesis, an essential anabolic process required for cell growth and proliferation, primarily through the phosphorylation of ribosomal protein S6 kinases 1 and 2 (S6K1/2) and eukaryotic translation initiation factor 4E (eIF4E)-binding proteins 1 and 2 (4E-BP) (Fig. 1.1). Phosphorylation of 4E-BP disrupts its engagement with eIF4E at the 5' ends of mRNAs, an interaction that in a basal state inhibits translation initiation[5]. Upon release from 4E-BP, eIF4E recruits eIF4G to assemble a larger translation initiation complex on the mRNA, including the small (40S) subunit of the ribosome. mTORC1 also promotes acute stimulation of mRNA translation through its phosphorylation and activation of S6K1/2, which subsequently phosphorylates multiple downstream translation factors[6]. mTORC1 preferentially stimulates the translation of mRNAs encoding ribosomal proteins and translation initiation and elongation factors, all of which contain a 5'terminal oligopyrimidine tract (TOP) or 5'-TOP-like sequence at the start of its 5' UTR[7]. The molecular details of this selectively are still under active investigation. Taken together, the processes regulated by the S6Ks and 4E-BPs promote a global increase in the capacity of cells to synthesize protein.

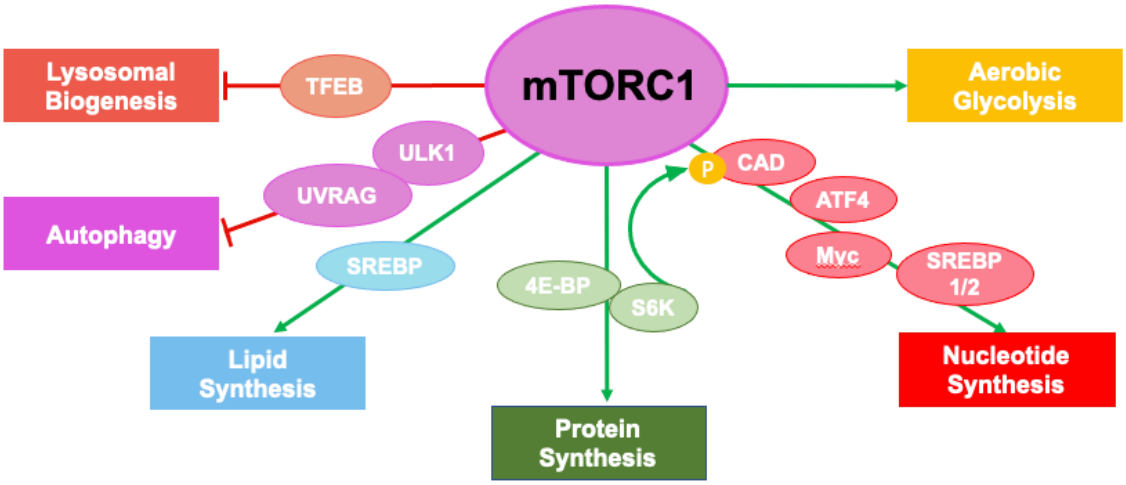


Figure 1.1 Anabolic and catabolic processes downstream of mTORC1 supporting growth and proliferation.

When activated, mTORC1 drives lipid synthesis through SREBP and protein synthesis via 4E-BP and S6K. Through S6K mediated phosphorylation of CAD, and the transcription factors ATF4, Myc and SREBP1/2, mTORC1 drives nucleotide synthesis. mTORC1 promotes a shift to aerobic glycolysis to further support its anabolic program. mTORC1 inhibits catabolic processes such as lysosomal biogenesis and autophagy through TFEB, and ULK1 and UVRAG, respectively.

mTORC1 also regulates the production of nucleotides, a second anabolic process that is required for proper cell growth (Fig. 1.1). Downstream of mTORC1, the S6K1-mediated phosphorylation of the enzyme carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, dihydroorotase (CAD), which catalyzes the first three reactions in the *de novo* pyrimidine synthesis pathway, stimulates an increase in its activity, thereby linking pro-growth signals upstream of mTORC1 to the biosynthesis of UTP, CTP, dTTP and dCTP[8,9]. mTORC1 also promotes the *de novo* synthesis of purines, in a more delayed manner, through downstream activation of the transcription factors ATF4, Myc, and the sterol regulatory element-binding proteins (SREBP1/2), all of which induce the expression of specific metabolic enzymes within or contributing to the de novo purine synthesis pathway[10].

A third major anabolic process controlled by mTORC1 is de novo lipid synthesis (Fig. 1.1). The SREBP family of transcription factors are master regulators of de novo lipid synthesis and upon activation by mTORC1 they induce the expression of a host of enzymes involved in both fatty acid and sterol synthesis[11-14]. Briefly, sterol depletion is the canonical signal that activates SREBP, whereby it moves from the endoplasmic reticulum to the Golgi apparatus for processing. A mature and active form of SREBP then enters the nucleus to induce the expression of sterol regulatory elements. mTORC1 primarily activates SREBP by promoting its processing and nuclear localization through a still unresolved mechanism in which the mTORC1 substrates S6K1 and Lipin1, and perhaps other mTORC1 targets, have been implicated[11,12,15,16]. Additionally, the splicing and maturation of SREBP-dependent transcripts encoding lipogenic enzymes has been shown to be regulated by both mTORC1 and S6K1 through the activation of SR protein kinase 2 (SRPK2), which regulates RNA splicing factors[17].

Aside from its primary role in stimulating protein, nucleotide, and lipid synthesis, mTORC1 regulates additional metabolic processes to further support its anabolic program. Through these mTORC1 dependent mechanisms, a shift from oxidative phosphorylation to aerobic glycolysis, also known as the Warburg effect, takes place within proliferating cells (Fig. 1.1). This shift towards glycolysis is believed to support anabolic metabolism by maintaining redox balance and promoting the use of glycolytic intermediates for critical side branches such as serine/glycine synthesis and the pentose-phosphate pathways. These pathways, in turn, provide essential precursors such as ribose, glycerol, non-essential amino acids and one-carbon units for the synthesis of proteins, nucleotides, and lipids[11,18].

In conditions of nutrient shortage or cellular stress, bulk autophagy provides an influx of nutrients through the recycling of macromolecules or organelles via their degradation in the lysosome. As such, in parallel to its role in promoting anabolic processes, mTORC1 suppresses this important catabolic process as well as lysosomal biogenesis to inhibit the turnover of macromolecules (Fig. 1.1). The best described mechanism of autophagy suppression by mTORC1 is through its phosphorylation and inhibition of Unc51-like kinase 1 (ULK1), a key regulator of the early phases of autophagy induction[19-21]. mTORC1 also suppresses the later stages of autophagy through its inhibition of UV radiation resistance-associated gene product (UVRAG)[22]. Finally, though not exhaustively, the transcription factor EB (TFEB) is a key inducer of the global expression of genes encoding both the autophagy machinery and degradative components of the lysosome. Direct phosphorylation of TFEB by mTORC1 causes it to be sequestered to the cytosol where it can no longer initiate its transcriptional program, likely contributing to the long term inhibitory effect of mTORC1 signaling on both autophagy and lysosome biogenesis[23-26].

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

Growth signals in the form of nutrients, energy, growth factors, cytokines and hormones converge on mTORC1 regulation through distinct signaling cascades. Two systems of Ras-related small GTPases (or G-proteins), and their regulators, lie directly upstream of mTORC1 and coordinate a vast network of signals to activate mTORC1 on the cytosolic face of the lysosome[27-31] (Fig. 1.2). The family of Rag GTPases (RagA, RagB, RagC, and RagD) form a heterodimer of RagA or B bound to Rag C or D, and in their activated state recruit mTORC1 to the surface of the lysosome[27,32]. The guanylate-loading state of the Rag GTPases is dictated by several multi-protein complexes that sense intracellular amino acids and promote the activated conformation of the Rag GTPases in nutrient replete conditions. The activated Rag GTPase heterodimer, bound to a protein complex referred to as the Ragulator, serves as a docking site for mTORC1 at the lysosome where it is subsequently activated by the GTPase Ras homolog enriched in brain (Rheb)[32].

Rheb is a ubiquitously expressed protein found on multiple endomembrane compartments. A sub-pool of Rheb is localized to the lysosome, and in its activated state is an essential activator of mTORC1[33,34]. The activation state of Rheb is directly controlled by the tuberous sclerosis complex (TSC) protein complex. The TSC complex is comprised of the TSC1 and TSC2 tumor suppressors and a smaller protein, TBC1D7[29,35,36]. Major growth factor signaling pathways converge on the TSC complex and act to inhibit its ability to regulate Rheb primarily through phosphorylation events on TSC2[2]. One such mechanism by which the TSC complex is inhibited is its dissociation from the lysosome following phosphorylation[34]. Loss of

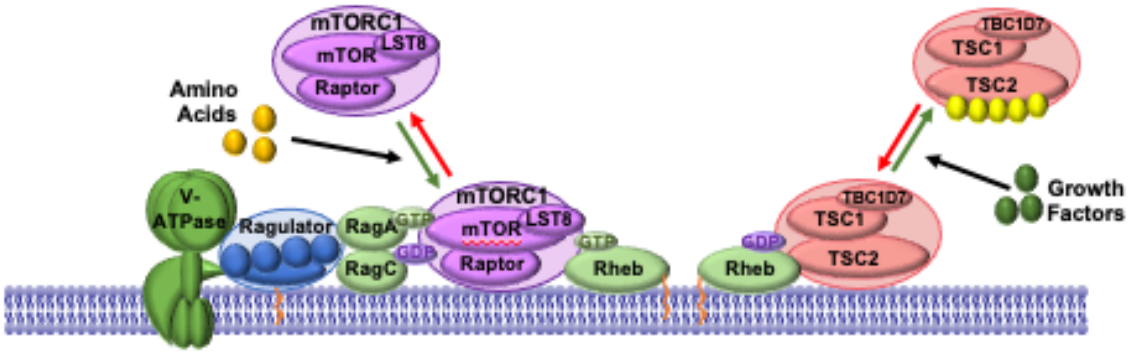


Figure 1.2 Integrated regulation of mTORC1 by growth factors and amino acids at the lysosome. In response to amino acids, mTORC1 is recruited to the surface of the lysosome via an activated Rag GTPase heterodimer. The Ragulator protein complex engages the Rags to support tethering of mTORC1 at the lysosome. Once at the lysosome, mTORC1 can be directly activated by the GTPase Rheb, a small pool of which resides on the lysosome. Rheb is under the direct negative control of the TSC complex, which only localizes to the lysosome to inhibit Rheb in the absence of growth factors. In response to growth factors, the TSC complex is phosphorylated and is released from the lysosome, allowing Rheb to become active and turn on mTORC1 signaling.

the TSC complex at the lysosome relieves its negative regulation of Rheb, allowing Rheb to directly activate mTORC1 to promote downstream anabolic processes.

The molecular events leading to full mTORC1 activation act as an "AND-gate", in which mTORC1 signaling is only turned on when both the Rags and Rheb are activated through distinct upstream signals. Although nutrients are required to activate mTORC1, they are only sufficient for its basal activity. Full activation of mTORC1 and its downstream anabolic program requires the presence of both nutrients and growth factors[6]. Regulation of the Rags and Rheb by their upstream regulatory proteins is, therefore, key to coordinating the activation state of mTORC1 under constantly fluctuating growth conditions. As such, a mechanistic overview of the GTPase cycle and GTPase regulators will be described in the following section.

GTPases and their regulators (GEFs, GAPs & GDIs)

GTPases & The GTPase cycle

GTP-binding hydrolases (or GTPases), also called G-proteins, serve as signaling switches through their interconversion between GTP- and GDP-bound states. A superfamily of Ras-related small G-proteins regulate a diversity of intracellular processes ranging from cell growth and cell differentiation to vesicular and nuclear transport. The Ras superfamily is divided into five major branches based on amino acid sequence and functionality: Ras, Rho, Rab, Ran and Arf[37]. GTPases share the common mechanism of acting as a molecular switch that toggles between "on" and "off" signaling states, referred to as the GTPase cycle (Fig. 1.3). In the GTP-bound form, the GTPase is "on" and capable of engaging its downstream effectors. In the GDP-bound state, the GTPase is "off" and is unable to engage its effectors, thereby blocking further propagation of a given signal[38].

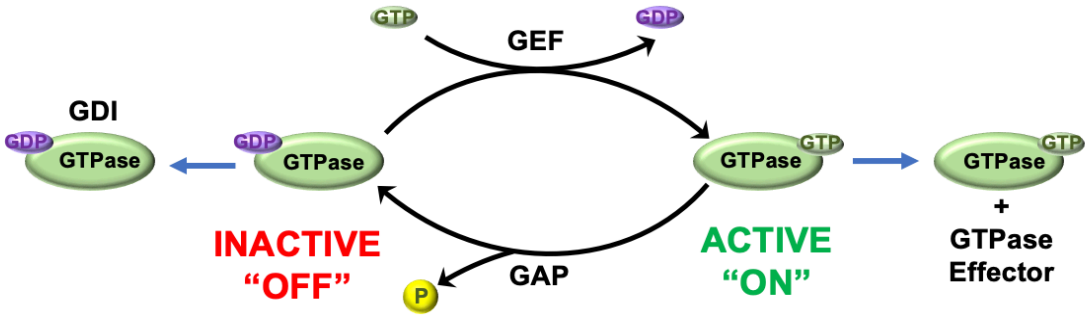


Figure 1.3 The GTPase cycle. GTPases act as a molecular switch, toggling between an activated, GTP-bound state and an inactive, GDP-bound state. GAP proteins stimulate intrinsic GTPase activity of GTPases, promoting the inactive form of a GTPase. GEF proteins release nucleotide (GDP) and enable exchange for GTP, promoting the active form of a GTPase. A third regulatory class of proteins, GDIs, maintain GTPases in their inactive form.

Fundamental to all GTPases is their intrinsic ability to hydrolyze GTP to GDP, enabling them to behave as molecular switches[39]. With few exceptions, GTPases have equally high affinity for both GTP and GDP, with binding constants typically in the picomolar to nanomolar range. Given the high affinity for nucleotide, GTPases are rarely, if ever, found in their nucleotide-free state[39]. While in the GTP-bound conformation, GTPases send a sustained and direct signal to keep the biological function to which they control in the "on state". As such, mutations that impair GTP hydrolysis prolong the activation state of a GTPase and lead to hyperactivation of processes, such as occurs commonly in cancer with K-Ras[40].

GTPases interact with a variety of effectors in distinct intracellular localizations, which accounts for their functional specificity. However, all Ras-related small GTPases share the same three-dimensional domain called the G-domain. The basic function of nucleotide binding and hydrolysis is carried out in this roughly 20-kD domain and makes up most of the GTPase structure[38]. The G-domain is formed by a six-stranded β–sheet flanked by five α-helices and contains five highly conserved sequence elements (G1-G5) surrounding the nucleotide binding site. The most important motifs for tight nucleotide binding are the interactions between the guanine base and the G4/G5 motifs and G1 with the β-phosphate. The GXXXXGKS/T motif (G1) is a conserved phosphate binding loop (or P loop) that interacts with the β and α-phosphates of the nucleotide. The second motif (G2) is in switch I not well conserved outside of a threonine residue. The Asp side chain from the DXXG motif (G3) coordinates a magnesium (Mg^{2+}) ion, which is essential for tight nucleotide binding and GTP hydrolysis. The Asp side chain of the N/TKXD (G4) motif interacts with the nucleotide base and drives guanine specificity, along with the weakly conserved EXSAK motif (G5)[41].

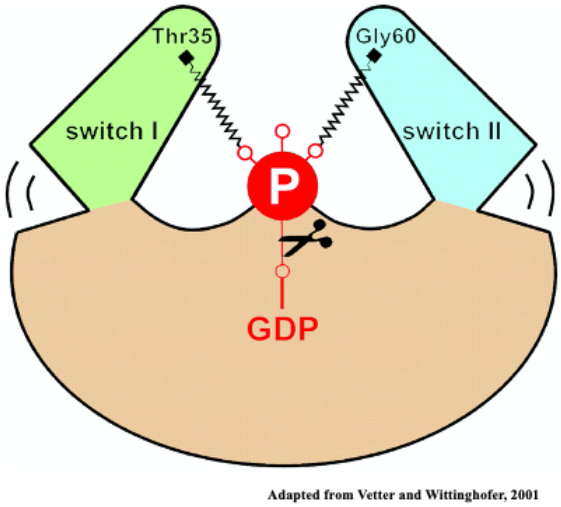


Figure 1.4 The "Loaded Spring Mechanism" of GTP hydrolysis. A canonical spring mechanism of GTP hydrolysis involves hydrogen bonds between the γ-phosphate of GTP and a conserved Thr in switch I and Gly in switch II. Release of these interactions occurs during GTP hydrolysis and "relaxes" the switch regions into the GDP bound confirmation.

The P-loop, switch I and switch II domains form the active site for GTP hydrolysis and enable GTPase effector and regulator binding[42]. When bound to either GDP or GTP, GTPases have similar confirmations but differ in their "switch regions". While GDP bound, most GTPases show a large variation in their structure, but the GTP bound forms are generally more alike. In the GTP-bound form, two hydrogen bonds from the γ-phosphate oxygens are formed with the main chain amine groups of conserved Thr and Gly residues, located on switch I and switch II respectively (Thr^{35} and Gly^{60} in Ras). The glycine residue is part of the DXXG motif (G3), while the Thr is the conserved G2 domain. The confirmational change from GTP to GDP has been described as a "loaded-spring" mechanism whereby release of the γ-phosphate after hydrolysis relaxes the switch regions into a GDP-specific conformation[38] (Fig. 1.4). Although this is a universal mechanism, the extent of conformational change varies between the different GTPases and some require additional elements. Moreover, the precise switch region residues need to be individually determined for the distinct proteins[38].

Following the G-domain, most GTPases end with a short sequence at their c-terminus that undergo post-translational lipid modifications (prenylation) and proteolysis. These modifications act as lipid "anchors" and are required for the proper subcellular membrane localization of a GTPase. The majority of Ras and Rho proteins end with a specific motif referred to as the CAAX box (C=Cysteine, A=aliphatic amino acid, X=methionine or serine). The first modification occurs when a farnesyltransferase (FTase) or geranylgeranyl transferase (GGTase) enzyme catalyzes the covalent addition of a 15-carbon farnesyl isoprenoid or a 20-carbon geranylgeranyl isoprenoid, respectively, to the cysteine. Following this first reaction, the -AAX is cleaved by peptide proteases and the new C-terminal carboxylate is

carboxymethylated[43]. The enzymes catalyzing these modifications reside on the cytosolic face of the endoplasmic reticulum (ER).

GTPase regulators: GEFs, GAPs & GDIs

Although varying between different family members, small GTPases generally have high affinity for both GDP and GTP (lower nanomolar to picomolar range), slow nucleotide dissociation rates (half-lives on the order of one or more hours) and weak intrinsic GTP hydrolysis rates. Given that biological processes must be regulated on a significantly faster timescale, the GTPase cycle is modulated by two main classes of regulatory proteins that exist to accelerate nucleotide binding, exchange, and hydrolysis: Guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). Fundamentally, GEFs turn on GTPase signaling while GAPs turn off GTPase signaling. GEFs and GAPs are generally regulated by cellular signaling events to control the GTPase cycle[44].

GEFs facilitate the exchange of GDP for GTP by increasing the rate of nucleotide dissociation by several orders of magnitude[38]. When GEFs engage a GTPase, the nucleotide-binding pocket is modified such that nucleotide affinity is lowered, thus allowing for nucleotide dissociation. A series of fast reaction steps make up the GEF reaction, which goes from a binary and low affinity complex formed between the GTPase, nucleotide and GEF to a binary and stable nucleotide-free complex. Free nucleotide can subsequently bind the empty nucleotide binding pocket on the GTPase. Because of the reversibility of the GEF reaction, GEFs, in principle, act as catalysts to increase the rate at which equilibrium between a GDP and GTP bound GTPase is reached[38]. The equilibrium is pushed towards the GTP bound state as the concentration of

intracellular GTP is ten times more abundant than GDP. Upon binding of the GTP, the affinity of the nucleotide bound GTPase for the GEF is lowered and it is subsequently displaced[44].

The catalytic domain and exact mechanism of GEFs varies between families, however, they all work under the same principle of inducing a conformational change in the switch regions and P loop of the GTPase. This interaction deforms the GTPase nucleotide binding site to displace the bound nucleotide, thereby reducing nucleotide affinity[38,44]. Specifically, GEFs first take advantage of the flexible switch 1 region and project it away from the nucleotide-binding site through steric hindrance. Second, GEFs make extensive contacts with the switch 2 domain of a GTPase, which likely stabilizes the nucleotide free form of a GTPase (a highly unstable form). In addition to these general mechanisms, some GEFs will insert acidic residues into the phosphate-binding site which helps to expel the bound nucleotide. Others will sterically occlude the Mg^{2+} binding site directly, and some GEFs remodel the GTPase switch II domain such that a methyl group from a conserved alanine will project towards the Mg^{2+} binding site and contribute to hydrophobic repulsion of the Mg^{2+}[45]. In the final step of a GEF reaction, the base of the incoming nucleotide binds first as the GEF is displaced[44].

GEFs are regulated primarily through intramolecular autoinhibitory interactions. Crystallization of GEF catalytic domains on their own or in complex with their target GTPase have indicated that the catalytic domain remains largely unchanged in the different states. Instead, domains outside of the catalytic domain appear to obstruct access to the GTPase-binding site and only through large conformational changes of these domains is GEF activity promoted[45].

The second essential regulator in the GTPase cycle, GAPs, enhance the intrinsic hydrolysis rate of GTPases, accelerating the rate of conversion from GTP to GDP by many orders of magnitude. As with GEFs, the exact mechanism of catalysis is not conserved and

various ways to enhance GTPase activity is utilized. Early insights into GAP mechanisms came from structural and biochemical studies of the Ras-RasGAP complex. First, RasGAP coordinates the attacking water in the GTP hydrolysis reaction by stabilizing the position of Gln 61 in switch II of Ras. Next, RasGAP positions an arginine residue, called an arginine finger, into the phosphate binding site of Ras. This insertion neutralizes the negative charge of the GTP γ-phosphate to stabilize the transition state[46]. Similar mechanisms have subsequently been determined for additional GAPs, which vary by their catalytic residue: Ras and Rho GTPases have arginine-finger GAPs[46], Rab GTPases have arginine/glutamine dual-finger GAPs[47] and Rap, Ral and Rheb GTPases have aspargine-thumb GAPs[48-51]. Despite differences in catalytic residue type and orientation, GAPs generally function to stabilize the intrinsic catalytic machinery of a GTPase and insert a catalytic residue in *trans,* thereby stimulating GTP hydrolysis[44].

GAP regulation is essential for maintaining proper signaling downstream of GTPases. Similar to GEFs, structural studies of GAP domains have indicated that the activity of a GAP is not regulated by conformational changes within the GAP domain. Instead, other auxiliary domains on a GAP are involved in spatiotemporal regulation. While GAP regulation is still being investigated, membrane-interacting domains that recruit GAPs to membranes containing their GTPase targets have been shown for the RhoGAP, B2-chimerin and ArfGAP1[52,53]. Similar mechanisms are likely at play for many other GAPs. In the context of the mTORC1 signaling pathway, the TSC complex acts as a GAP for Rheb. The specific mechanism of its catalytic activity, and regulation, will be discussed in a later section.

Some GTPases are modulated by a third class of proteins called guanine nucleotide dissociation inhibitors (GDIs). The GDIs that have been thus far characterized engage the

prenylated C-terminal end of a GTPase, extract it from the membrane to which it is inserted, and shield the hydrophobic tail from the aqueous cytosolic environment[44]. Through these steps, GDIs function to prevent the dissociation of GDP and maintain them in a soluble and inactive complex. Thus far, three families of this class of regulators have been identified: RhoGDI, RabGDI and Phosphodiesterase-δ (PDEδ). The Rho and Rab GDIs recognize the geranylgeranylated Rho and Rab proteins, respectively, while PDEδ binds specifically to different classes of farnesylated GTPases[45]. While GDIs prevent the exchange of GDP, it has now been established that their main function is to maintain their target GTPase in a soluble and inactive complex. PDEδ now falls into the GDI category for this reason[45].

Both Rho and Rab GDIs have two distinct domains, one of which recognizes the GTPase core while the other recognizes the geranylgeranylated C-terminus. While the GTPase core and lipid binding domains are structurally unrelated between the two families, these domains are positioned far away from each other, suggesting that these GDIs interact with their GTPases in a stepwise fashion[54,55]. Interestingly, the RhoGDI has been shown to associate with both GDP and GTP bound Rho, while the RabGDI interacts specifically with Rab-GDP[45]. PDEδ is a small protein (17 kDa), and structurally related to the lipid binding domain of RhoGDI[56]. This protein differs from the other two GDIs in that it does not contain a GTPase core recognition domain, and only associates with GTPases through the farnesylated C-terminus. It is likely this reason why PDEδ has a relaxed specificity that extends to unrelated proteins[57].

Unlike GEFs and GAPs, mechanisms of GDI regulation are still under investigation. It is thought that GDI-displacement factors (GDFs) for RabGDI and PDEδ work by displacing the lipid tail such that it is “squeezed” out through shrinkage of the lipid binding pocket[45]. Conversely, no GDFs have been proposed for Rho-GDI and it is instead regulated by

phosphorylation. Phosphorylation on specific sites displace RhoGDI from its GTPase and bring it into proximity to a membrane for GTPase depositing[58].

Not surprisingly, many missense mutations have been found in the genes encoding GEFs, GAPs and GDIs that contribute to a variety of human diseases, highlighting the importance of their regulatory role in the GTPase cycle. Many structures of these proteins are available and have enabled the mapping of missense mutations onto structural elements. In doing so, the mutations can be associated with the biochemical function of which they disrupt. For example, mutations in the catalytic domain of GAPs reduce GAP activity and thereby prevent the downregulation of GTPase signaling, gain of function mutations in GEFs can promote sustained GTP-loading and GDI mutations have been shown to impair GTPase extraction from membranes[45,59-61]. Of relevance to this dissertation, numerous missense mutations in the TSC complex can lead to the disease tuberous sclerosis complex (TSC), which will be reviewed in a later section.

Upstream regulation of mTORC1: The amino acid sensing branch

The Rag GTPase heterodimer

The heterodimeric Rag GTPase is central to the amino acid sensing and processing network upstream of mTORC1[62]. One functional unit of the Rag heterodimer is formed when RagA or RagB (RagA/B) tightly engages RagC or RagD (RagC/D)[63,64]. The active confirmation of this subunit occurs when RagA/B binds GTP and RagC/D binds GDP. In this state, the Rag GTPases interact with the raptor subunit of mTORC1 and recruit the complex to the lysosome[27,65,66]. In the inactive confirmation, RagA/B binds GDP while RagC/D binds GTP. This state repels mTORC1 into the cytosol and effectively shuts off the amino acid sensing

pathway into mTORC1[27]. The Rag GTPases do not contain any lipidation motifs and localize to the lysosome through their engagement with Ragulator, a pentameric complex[32,67].

The nucleotide loaded state of the Rag GTPases occurs through extensive crosstalk between the Rag subunits within the heterodimer. When a Rag subunit is loaded with GTP it becomes dominant and controls the loading status of the second subunit either by 1) preventing the binding of GTP to the second subunit or 2) stimulating hydrolysis to GDP if GTP does spontaneously bind to the second subunit. The intrinsic GTP off rate and hydrolysis rate of the Rag GTPases are extremely slow, which enables the Rag heterodimer to remain locked in either an inactive or active state, thereby sending a direct and stable signal to mTORC1. Dual binding of the Rag heterodimer to either GDP or GTP is considered an unstable state, and the function of this loading state is currently unknown[68].

Regulators of the Rag GTPase heterodimer

An intricate network of regulatory proteins act to control the loading state of the Rag GTPases (Fig. 1.5). These protein complexes ensure that the Rag GTPases can quickly toggle between their active and inactive state in response to nutrient availability and fluctuations. mTORC1 senses both intra-lysosomal and cytosolic amino acids, and regulators of the Rag GTPases reside within these two branches of the nutrient-sensing pathway[69].

Lysosomal amino acids are sensed through a mechanism that requires the vacuolar H+-adenosine triphosphate ATPase (V-ATPase) and SLC38A9. Both transmembrane proteins positively regulate mTORC1 signaling and have been shown to interact with Ragulator. In addition to its role in lysosomal tethering of the Rag GTPases, Ragulator also serves as a GEF for RagA/B, promoting the exchange of GDP for GTP. SLC38A9 is a direct sensor of intra-

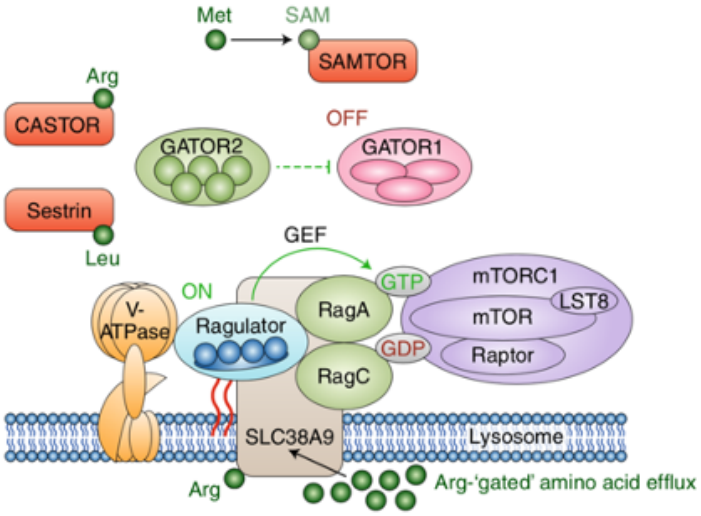


Figure 1.5 Amino acid sensing at the lysosome. In the presence of amino acids, mTORC1 is recruited to the lysosomal surface via an activated RAG GTPase heterodimer and its association with Ragulator. Leucine, arginine and SAM (derived from methionine), directly bind Sestrin, Castor and Samtor, respectively, thereby inhibiting GATOR1 through GATOR2. Through intralumenal arginine sensing, SLC38A9 mediates the release of leucine and other amino acids into the cytosol. GEF activity towards RagA/B by Ragulator and SLC38A9 is also promoted.

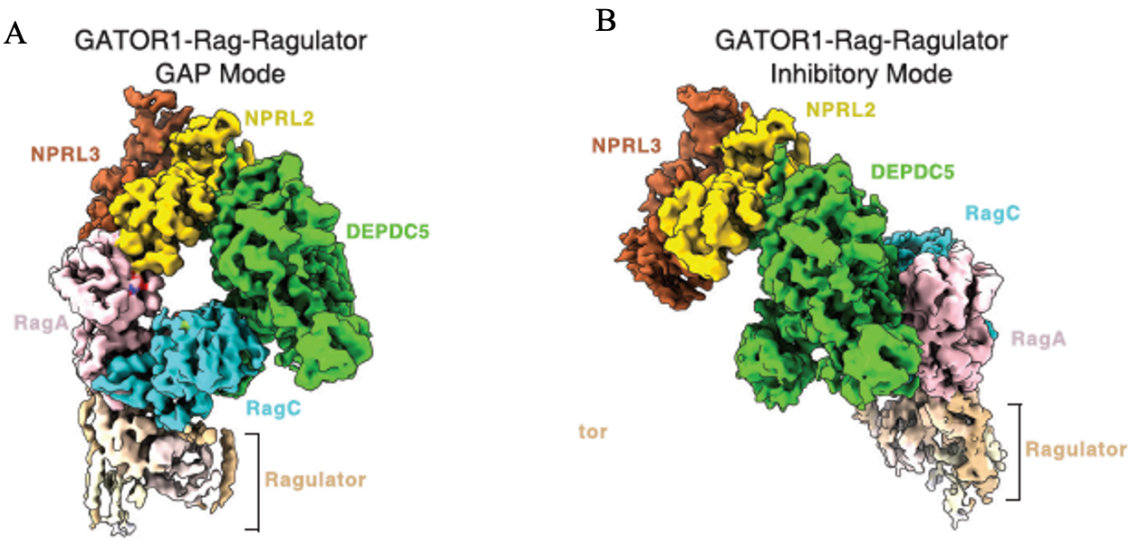
lumenal arginine and transports leucine and other neutral amino acids out of the lysosome while also working in concert with Ragulator as a second GEF for RagA/B. Thus, SLC38A9 works both directly, as a GEF, and indirectly, through cytosolic release of leucine, to promote RagA/B-GTP loading[70-72]. More recently, Ragulator has been shown to function as a non-canonical GEF towards the Rag Heterodimer and specifically accelerates the release of GTP but not GDP from RagC/D[73].

Sestrin2, CASTOR1 and SAMTOR sense cytosolic leucine, arginine and S-adenosylmethionine, respectively and signal through the 'GAP activity towards Rags 1' (GATOR1) and GATOR2 protein complexes[74-76]. GATOR1 is tethered to the lysosomal surface by the KICSTOR complex and serves as negative regulator of mTORC1 signaling. Under low nutrient availability, GATOR1 acts as a GAP towards RagA/B, promoting inactivation of the Rags[77-79]. Conversely, GATOR2 acts as a positive regulator of mTORC1 through its inhibition of GATOR1 at the lysosome, the molecular mechanism of which is currently unknown. GATOR2 is inhibited by its interaction with CASTOR1 and Sestrin2. When these two amino acid sensors bind arginine and leucine, respectively, a conformational change causes them to dissociate from GATOR2. Upon this dissociation, GATOR2 interacts with and inhibits GATOR1 thereby promoting GTP loading on RagA/B and subsequent recruitment of mTORC1 to the lysosome[74,75,80,81]. mTORC1 also senses methionine levels through an intermediate in the methionine cycle, S-adenosylmethionine (SAM). SAM binds SAMTOR which directly inhibits GATOR1[76]. A second identified GAP in this highly intricate amino acid sensing molecular network is the Folliculin-FNIP2 complex, which acts on RagC/D, to promote Rag activation in the presence of amino acids[82]. A detailed overview of GATOR1, the parallel GAP to the TSC complex, will be provided in the following section.

Detailed overview of GATOR1

Cryo-EM structural models have revealed that GATOR1 binds the Rag GTPase heterodimer in two modes: A GAP mode and an inhibitory mode (Fig. 1.6). Interestingly, this GAP-independent binding mechanism has yet to be described for other GAP proteins. GATOR1 is a heterotrimeric complex consisting of DEP domain-containing protein 5 (DEPDC5), Nitrogen permease regulator 2-like protein (NPRL2) and nitrogen permease regulator 3-like protein (NPRL3). The first cryo-EM model of GATOR1 captured it in the inhibitory mode as the GAP mode, initially identified through genetic approaches, has 40-fold weaker affinity for the Rag GTPase and could not be captured for structural studies[83]. More recently, the Cryo-EM structure of GATOR1 in the GAP mode was resolved using mutants of the Rag GTPase heterodimer and GATOR1 that increase the affinity of the GAP mode interaction[84]. In the inhibitory mode, GATOR1 binds to the nucleotide binding pocket of RagA/B in its GTP-bound confirmation through the B strand of DEPDC5 called the "critical strip", no contacts between GATOR1 and RagC/D are observed. In this conformation, the GAP domain of GATOR1 does not engage the Rag GTPases and GTP hydrolysis is 30-fold lower than in the GAP mode. It is suggested that the purpose of the inhibitory binding mode is to prevent hyperactivation of GATOR1 to keep mTORC1 in an active state.

The structure of GATOR1 bound to the Rag GTPases in the GAP mode revealed extensive contacts between all three of the subunits of GATOR1. The GAP interface is formed between the heterodimerized longin domains of NPRL2-NPRL3 and the NBD of RagA/B while an auxiliary interface is formed between RagC/D and the N-terminal domain (NTD) and C-terminal domain (CTD) of DEPDC5. The structure of GATOR1 in the GAP mode also confirmed that Arg78 of NPRL2 acts as the catalytic arginine finger to stimulate GTPase activity



Adapted from Egri et al., 2022

Figure 1.6 GATOR1 binds the Rag GTPase heterodimer in two modes. (A) GATOR1 binding to the Rag GTPase heterodimer in the GAP mode. (B) GATOR1 binding to the Rag GTPase heterodimer in the inhibitory mode.

of RagA/B[83-85]. It is currently unknown how GATOR1 integrates signals to switch from an inhibitory to gapping state and further biochemical studies will likely reveal this important mechanism.

Upstream regulation of mTORC1: the growth factor sensing branch

Overview

Like amino acid sensing pathways, signals from growth factors and hormones are also integrated at the lysosome to activate mTORC1 (Fig. 1.7). These signaling pathways converge on the TSC complex, the sole established regulator of Rheb. The PI3K-Akt and Ras-ERK pathways are two major growth factor signaling pathways known to impinge on the TSC complex. Activation of the PI3K-Akt pathway occurs upon binding of insulin or insulin-like growth factor 1 (IGF1) to cell surface receptor tyrosine kinases (RTKs). RTKs stimulate phosphoinositide 3-kinase (PI3K), a lipid kinase, which phosphorylates phosphatidylinositol-4-5-bisphosphate (PIP_2) to produce phosphatidylinositol-3-4-5-bisphosphate (PIP_3)[86]. Akt is recruited to the plasma membrane by PIP_3 where it is phosphorylated at T308 by membrane-bound phosphoinositide-dependent kinase (PDK1), leading to partial activation[87]. Secondary phosphorylation at S473 by mTORC2 results in complete Akt phosphorylation[88]. Upon activation, AKT phosphorylates several proteins containing the RXXS/T motif to regulate biological processes[89]. The TSC complex has many known and predicted Akt phosphorylation sites and upon multi-site phosphorylation by Akt it is functionally inhibited[90,91]. Mechanistically, phosphorylation by AKT at these sites causes the TSC complex to dissociate from the lysosome, enabling Rheb to become GTP-loaded and activate mTORC1[34].

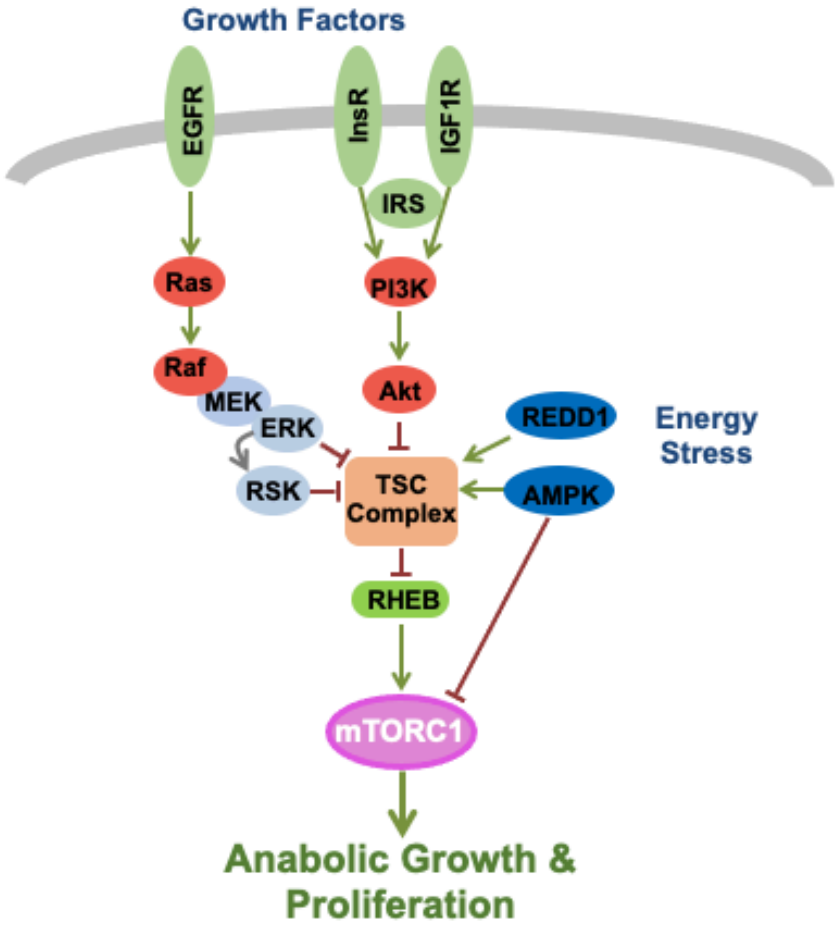


Figure 1.7 The growth factor activated signaling pathways. The PI3K-Akt and Ras-ERK pathways are two major growth factor signaling pathways known to impinge on the TSC complex. The PI3K-Akt pathway is activated upon binding of insulin/IGF1 to RTKs while the Ras-ERK pathway is activated upon binding of EGF to RTKs.

mTORC1 is also regulated by the Ras-ERK pathway through TSC complex phosphorylation. Upon binding of the growth factor epidermal growth factor (EGF) to cell surface RTKs, the receptor is auto-phosphorylated and recruits and stimulates the Ras GTPase[92]. Activated Ras recruits and activates Raf at the plasma membrane which then phosphorylates the mitogen-activated protein kinase (MAPK) kinase MEK. Phosphorylated MEK subsequently phosphorylates the extracellular signal-regulated kinase (ERK), a kinase with many substrates involved in cell cycle progression and growth. One such substrate in this signaling cascade is the cytoplasmic protein p90 ribosomal S6 kinase (RSK), which also phosphorylates many downstream targets[93]. Like Akt, site-specific phosphorylation on TSC2 by both ERK and RSK leads to inactivation of the TSC complex and activation of mTORC1 by Rheb[30,90,91,94,95]. The mechanism of TSC complex inhibition by ERK and RSK has yet to be formally established, however unpublished data from the Manning lab demonstrates that phosphorylation of TSC2 by these kinases changes the lysosomal localization of the TSC complex.

Conversely, intracellular and environmental stressors such as hypoxia, low ATP levels and DNA damage negatively regulate mTORC1. Hypoxia and low cellular energy are sensed by AMP-activated protein kinase (AMPK), a metabolic stress regulator. AMPK indirectly suppresses mTORC1 through phosphorylation on the TSC complex at sites that activate the complex, and directly inhibits mTORC1 by phosphorylation of Raptor[96-98]. The Regulated in DNA damage and development 1 (REDD1) protein has also been shown to activate the TSC complex in response to hypoxic conditions[99]. Finally, DNA damage responsive pathways lead to increases in TSC complex activity through the induction of p53 target genes such as PTEN, the AMPK regulatory subunit (AMPKBβ) and TSC2[100]. Loss or mutation of TSC complex components or constitutive activation of the pathways that inhibit its activity, results in

uncontrolled levels of Rheb-GTP loading. In turn, high levels of Rheb-GTP promote aberrant mTORC1 signaling even in the absence of growth factors[6].

The direct mTORC1 activator, Rheb

Essential to mTORC1 activation is Rheb, a Ras-like small GTPase. Rheb is a 21 kDa monomeric protein belonging to the Ras superfamily of GTPases. Rheb, like other GTPases, acts as a molecular switch and directly binds to and activates mTORC1 when in its GTP-bound form[101]. Rheb is highly conserved from yeast to human, however it is not found in plants. Strikingly, early studies in *S. pombe* revealed that human Rheb can functionally replace Rhb1, the yeast homologue[102]. In lower eukaryotes, only one gene has been identified, whereas mammalian cells contain two Rheb genes: Rheb1 and Rheb2. Rheb1 and Rheb2 likely perform similar functions and differ in their tissue expression profiles. Rheb1 (from here on referred to as Rheb) is a ubiquitously expressed gene, while Rheb2 is limited to specific tissues[103].

Rheb is 184 amino acids long; the N-terminal GTPase domain makes up the first 169 amino acids, followed by a highly variable region (HVR) ending with a -CAAX motif. Rheb is farnesylated at the cysteine, followed by cleavage of -AAX by Rce1 and carboxymethylated by Icmt1[104]. The post-translational farnesylation on Rheb is required for its localization to endomembranes and specifically the lysosome[105,106]. Within the HVR, Rheb lacks palmitoylated cysteines or a polybasic domain, likely explaining why it does not localize to the plasma membrane, as is the case with Ras[104].

Rheb shares sequence homology with many other small GTPases but is particularly homologous to the Ras and Rap subfamily[105]. Several structures for Rheb (and Rheb mutants) bound to GDP, GTP and GppNHp (a non-hydrolyzable GTP analog) have been determined by

X-ray crystallography and NMR over the past 20 years[107-111]. These structures have revealed the major conformational changes that take place during the GTPase cycle and highlight the unique biochemical properties of Rheb. As previously described, the GTPase switch I and switch II regions are involved in the recognition and interaction with GTPase regulators (GAPs and GEFs) and their effectors. Switch I is particularly important for effector recognition and primary sequence and structural differences in this region drive effector specificity[38,112].

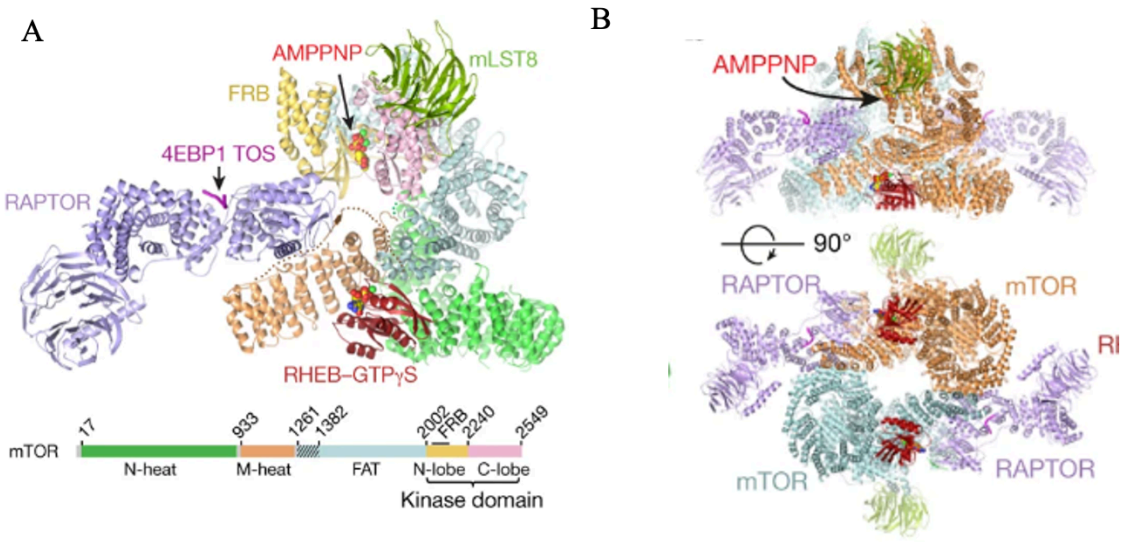
During the GTP to GDP transition, Rheb, like most GTPases, undergoes a conformational change in the switch I region (residues 33-41) and is highly flexible. While Ras and Rap undergo a similar confirmational change, the difference in confirmation between Rheb and these GTPases is more pronounced in the GDP bound form. The switch II region on Rheb (residues 63-79) is unique and drives the major structural differences between Rheb and other GTPases. While most GTPases adopt a long α-helical confirmation in switch II when bound to GTP or GppNHp, Rheb takes on an unraveled confirmation and forms an extended N-terminal loop that leads into a short helix. Importantly, this structure is maintained through the transition to Rheb-GDP, representing a distinctive structural property of Rheb that likely contributes to its unique biochemical properties such as unusually slow GTPase activity and a high activation state[109].

Given its unique structural differences, established catalytic residues on Ras have been shown to have no influence on Rheb activity and function. As such, Rheb has evolved a GTP hydrolysis mechanism, described as an autoinhibitory mechanism, that is entirely different from its close homolog, Ras. A single Tyr residue found in the switch I region is highly conserved but differs in its function among GTPases. In Rheb, Tyr35 contributes to autoinhibition by covering the nucleotide binding site and hindering intrinsic GTP hydrolysis, however this site is required

for GAP activity. Mutation of this residue to alanine (Y35A) results in a 10-fold increase in intrinsic GTPase activity. Further analysis of this mutant revealed that Rheb GTP hydrolysis requires Asp65 in switch II and Thr38 in switch I. Asp65 acts as the catalytic residue and activates a water molecule for nucleophilic attack in GTP hydrolysis. Of particular importance is the residue Gln64, which corresponds to the catalytic residue Gln61 in Ras. Gln64 is buried in a hydrophobic core and is unable to engage GTP or the catalytic active site on TSC2. As such, mutational studies have shown the Gln64 is dispensable to Rheb[110,113].

Mechanism of mTORC1 activation by Rheb

Recent structural determinations of Rheb bound to mTORC1 have elucidated mechanisms of mTORC1 activation. In the following section I have provided a high-level overview of mTORC1's structure before describing its interaction with Rheb. The cryo-EM structures of mTORC1 have revealed that the complex adopts a 2-fold symmetry forming a hollow rhomboid shaped core containing two copies of each subunit (mTOR, mLST8 and Raptor). The mTOR subunit has two contiguous HEAT repeat domains at its N-terminus, N-terminal HEAT (N-HEAT) and Middle HEAT (M-HEAT), followed by the FAT domain and the C-terminal Kinase domain. The kinase domain is formed by two lobes (N-ter and C-Ter), that make an active site cleft. Within the two lobes are the FKBP12-rapamycin Binding (FRB) domain found in the N-ter lobe and the LST8 binding element (LBE) residing in the C-ter lobe. As suggested by the name, LST8 binds to the LBE. The FAT domain adopts a C-shaped solenoid and acts as the organizing core of mTOR. Both ends of the FAT solenoid clamp onto the kinase domain N-ter and C-ter lobes, while the beginning of FAT packs with the start of the M-heat and the middle of FAT packs with the end of the N-heat. The mTORC1 dimer is formed through the



Adapted from Yang et al., 2017

Figure 1.8 Cryo-EM structure of mTORC1 activation by Rheb. (A) One copy of the RHEB–mTOR–RAPTOR–mLST8-4EBP1 complex with color coding corresponding to the linear schematic below. Rheb-GTYγS is Red. **(B)** Two angles of dimeric mTORC1 engaged to Rheb.

packing of the N-HEAT region of one mTOR with the M-HEAT regions of the second mTOR. RAPTOR binds to this interface creating a tripartite interface[33,114,115].

A Cryo-EM structure of Rheb-GTP in complex with mTORC1 indicates that Rheb forms a four-way interface with mTORC1 and binds the N-terminal portions of the N-heat, M-heat and FAT domains on mTORC1 (Fig. 1.8). Most of the contacts with mTORC1 are in the switch I and switch II regions; switch I binds to M-heat and FAT and switch II interacts with all three of the regions. A single Rheb molecule binds to each mTOR molecule within the mTORC1 complex. Rheb allosterically activates mTORC1 by binding roughly 60 Å away from the active site. Binding induces a significant confirmational change, primarily through the FAT domain on mTORC1, that brings mTORC1's active-site residues into the correct orientation for catalysis[33].

The TSC complex: a Rheb-GAP

The TSC complex is a heterotrimeric protein complex composed of the tuberous sclerosis complex (TSC) tumor suppressors TSC1 and TSC2, and a third subunit Tre2-Bub2-Cdc16 1 domain family member 7 (TBC1D7)[35]. TSC1 and TSC2, also referred to as hamartin and tuberin, are large proteins of around 130 and 200-kDa, respectively[116,117]. These proteins are generally not analogous to other proteins, apart from a 200 amino acid stretch at the C-terminus of TSC2. This domain resembles the GAP domain of Rap1GAP and is the region that possesses GAP activity towards Rheb[29,118,119]. TSC1 acts as a scaffold for TSC2 and TBC1D7, and collectively the three proteins form a stable, high-molecular weight protein complex. While TSC1 is found almost exclusively within the complex, a free pool of both TSC2 and TBC1D7 exists in cells, the function of which is unknown[34]. The mechanistic role of TBC1D7 within the TSC complex is also currently unknown, however a loss of TBC1D7 in cultured cells leads to a modest growth-

factor-independent activation of mTORC1. This activation is attributed to a reduction in GAP activity due to partial destabilization of the TSC complex. Importantly, the level of mTORC1 activation upon loss of TBC1D7 is substantially less than that observed upon loss of TSC1 or TSC2[35].

The TSC complex directly regulates Rheb at the lysosome, however, the precise molecular mechanism of TSC complex lysosomal localization remains elusive. In the absence of Rheb at the lysosome, either through complete depletion of Rheb in the cell or by inhibiting Rheb farnesylation, the TSC complex fails to localize to the lysosome[34]. This finding revealed that Rheb is required for TSC complex lysosomal localization. Several other proteins have also been suggested to play a role in its recruitment including the Rag GTPases, RhoB and G3BP1[120-122]. Within the TSC complex, a recent study describes an interesting model in which localization occurs through the N-terminal domain of TSC1 via its affinity for lysosomal phosphatidylinositol phosphate (PIP) lipids[123]. Whether or not TSC2 interacts with lysosomal lipids to tether the TSC complex to the lysosome is currently unknown.

It was originally believed that TSC2 phosphorylation by Akt inactivated the TSC complex by either turning off its Rheb-GAP activity or promoting disassembly or degradation of the functional TSC complex. However, it is now established that phosphorylation on TSC2 by Akt promotes the dissociation of the TSC complex from the lysosome[34]. Although the results are unpublished, findings from our lab have revealed that phosphorylation on TSC2 by ERK and RSK similarly regulate lysosomal localization of the TSC complex. Thus, lysosomal dissociation of the TSC complex represents a unifying mechanism of inhibition by the major growth factor-activated kinases impinging on TSC2—the molecular nature of this release event is still under investigation.

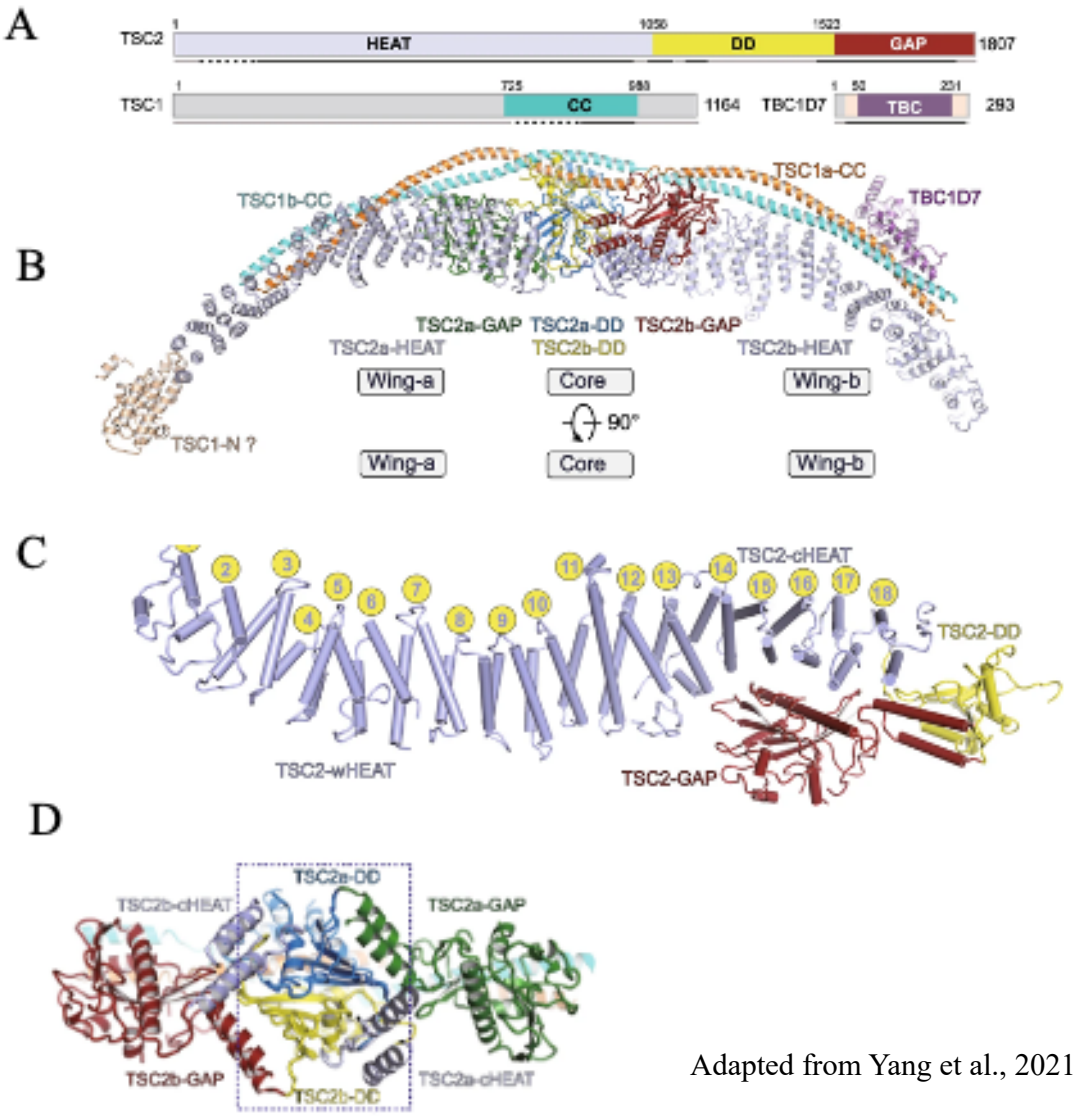
Structure of the TSC complex

Despite extensive studies conducted on the TSC complex function over the past several decades, a lack of structural insights into the complex have hampered a true mechanistic understanding of complex assembly and regulation of mTORC1 signaling. Until recently, only partial isolated domains have been resolved including TSC1 bound to TBC1D7[124,125], yeast N-terminal domains of TSC1[126], and the N-terminal domain and GAP domain of TSC2 from the thermophilic fungus *Chaetomium thermophilum*[127,128]. The two newly resolved cryo-EM structures of the full TSC complex are almost identical in overall shape and stoichiometry, with the Yang et al. structure providing enhanced resolution and side chain identification (Fig. 1.9). Despite providing only partial resolution of the complex, these structural data combined with the individual domain structures, have significantly enhancing our understanding of TSC complex stoichiometry and intra and intermolecular interactions[129,130].

The TSC complex is assembled in a 2:2:1 stoichiometry of TSC1, TSC2 and TBC1D7 with an asymmetrical organization. The overall structure exhibits an elongated arch shape fold upwards of 40 nm in length. Two TSC2 molecules (TSC2a/TSC2b) each form a seahorse-shaped conformation and interact through a dimerization domain in the central region of TSC2, forming the “core” of the protein complex. In each TSC2 molecule, the GAP domain folds back on itself and makes intramolecular contacts with the N terminal heat repeats. The two TSC1 molecules (TSC1a/TSC1b) are largely unresolved outside of the core Coil-Coil domain (CCs, residues 746-971) and intertwine in parallel to form an extended two helix bundle. The TSC1 dimer makes multiple contacts with the two TSC2 molecules, stabilizing the overall conformation of the complex. A single TBC1D7 is positioned at the C-terminal helices of the

TSC1 dimer (residues 937-971) and does not contact TSC2. The parallel dimerization of TSC1 combined with the recruitment of a single TBC1D7 generates a unique asymmetrical conformation of the complex[129].

Each TSC2 molecule consists of a HEAT repeat domain (HEAT), dimerization domain (DD), followed by a C-terminal GAP domain. 18 HEAT repeats make up the HEAT domain, the first 12 HEAT repeats (wHEAT) flank out of the central core and are stabilized by TSC1 while the following six HEAT repeats (cHEAT) interact with the DD and GAP domains. In both TSC2 molecules a pseudo-symmetric fold is formed by the cHEAT-DD-GAP domains while the wHEAT domains adopt distinct confirmations due to differences in their association with the TSC1 dimer. The TSC1 CC dimer makes four major contacts with TSC2: 1) Central region of TSC1a-CC (residues 800-890) with a “saddle” formed by the TSC2 DD dimer 2) N-terminal TSC1a-CC (residues 746-795) with a ridge formed by HEAT repeats 7-12 of TSC2a 3) C-terminal TSC1-CC dimer (residues 890-936) with HEAT repeats 8-12 of TSC2b and 4) C-terminal TSC1a-CC (residues 937-971) packed against a ridge of HEAT repeats 3-7 of TSC2b[129]. The last interaction between TSC2 and TSC1 confirms a known interaction between the N-terminus of TSC2 (1-418) and TSC1[127].



Adapted from Yang et al., 2021.

Figure 1.9 Cryo-EM structure of the TSC complex. (A) Color coded domain structure of the three complex components with un-resolved regions shown in grey (B) Resolved structure of the TSC complex (C) Cartoon model of a single TSC2 monomer, with each heat repeat indicated. (D) Close-up view of the intramolecular contacts within the dimerization interface.

The symmetrical core of the TSC complex is formed by the cHEAT-DD-GAP domains of the two TSC2 molecules and stable dimerization occurs through the DD domain of each TSC2 molecule. The DD domain consists of a four-stranded antiparallel β-sheet and five flanking α−helices. A saddle-shaped eight-stranded β-sheet is formed by the two β-sheets of each TSC2. Packed against the concave surface of the saddle is Dα5 (residues 1472-1483), which binds the other TSC2 molecule on HEAT18 and the following loop (residues 1024-1038). A loop leading into Dα4 inserts into a hydrophobic pocket within a three-way junction formed by the cHEAT-DD-GAP of the second TSC2. Lastly, The TSC1a CC domain is engaged by DD helices of both TSC2 molecules, further supporting TSC2 dimerization[129]. The GAP domain and N-terminal domain of TSC2 are highly conserved and have described TSC complex functions (complex stability and GAP activity, respectively). Interestingly, the middle region of TSC2, referred to as the DD domain by Yang et al, is less conserved and in some cases almost completely missing, as in the case of the S. pombe TSC2 orthologue[131]. This domain, in addition to its lack of conservation, is a serine/threonine-rich region that his heavily phosphorylated. The newly resolved structures have revealed its importance in TSC2 dimerization and a more detailed description of this region, and speculation into its functional significance, will be discussed in chapters two and three.

TSC2 GAP mechanism and interaction with Rheb

To date, the TSC complex is the only established regulator of Rheb, and this regulation is mapped exclusively to the GAP domain. The homology between TSC2 GAP and Rap1GAP is especially pronounced in the catalytic helix of the GAP domain. Rap1GAP uses an asparagine-thumb mechanism with residue Asn290 serving as the catalytic residue, which corresponds to

Asn1643 in TSC2[113]. Interestingly, several studies have shown that in the absence of TSC1, TSC2 is not functional *in vivo*[35]. In addition to gaining a better understanding of the overall conformation of the TSC complex, resolved structures from the last several years have collectively provided important mechanistic details of the GAP domain, its positioning within the TSC complex, and the interaction and catalytic mechanism of GAP activity towards Rheb. These studies largely support early biochemical and mutational analyses of Rheb and The TSC complex. It is important to note that to date, no structures of the TSC complex bound to Rheb exist and the structural details of this interaction are based exclusively on docking models that primarily use the Rap-Rap1GAP structure as a backbone[128,129].

The cryo-EM structure of the complete complex is well resolved in the GAP domain and indicates that the GAP domain is positioned within the core of the complex. The GAP domain of TSC2 adopts a characteristic mixed α/β-fold and consists of a central seven-stranded β−sheet stabilized by a long α helix (Gα5) that runs along the concave side. Packed against the convex side of the β−sheet is the catalytic helix (Gα3) and two loops (L1 and L2). In the context of the full complex, both catalytic cores are nearly identical in conformation with their catalytic pockets opened outwards. This positioning is coordinated by interdomain contacts between TSC2 GAP domain, HEAT domain, and DD domain as well as stabilizing interactions with TSC1[129].

Rheb docking models have confirmed that the N1643 residue of TSC2 acts as an asparagine thumb to accelerate GTP hydrolysis of Rheb and is positioned near Y35 of Rheb, which is likely the corresponding catalytic residue and in support of early biochemical studies. Other residues on the catalytic helix that are likely important for substrate binding include K1638 and R1639 which face towards Rheb, L1641 which faces in towards the core of TSC2 GAP and H1640 which putatively binds R15 of Rheb. Prior studies have shown that these sites are critical

for GAP activity[36,113,132] , and they are suggested to stabilize the conformation of the asparagine thumb[128,129].

These models have also revealed a unique recognition of Rheb by the TSC complex that differs from that of Rap-Rap1GAP and Ras-RasGAP binding patterns. First, L1 and L2 of the GAP domain are positioned near switch I and switch II on Rheb. Both alanine substitution and disease associated mutations within these loops (L1594 & L1597 on L1 and Q1665 & F1666 on L2) decrease GAP activity, suggesting that these loops support catalysis and substrate recognition. Second, a helix pair, GαN (residues 1525-1536) and GαC (residues 1739-1754) protrudes out of the catalytic core and is positioned near helices α2 and α3 of Rheb. These helices likely play a critical role in mediating the Rheb-TSC2 interactions as mutation of residues R1529, L1533 and the double mutation R1529/L1533[129] on GαN and R1749[133] on GαC results in a decrease in GAP activity.

Collectively, modeled interactions between Rheb and the GAP domain of TSC2 have provided an enhanced understanding of the important interactions required for the canonical GAP activity of the TSC complex. However, further structural studies of Rheb engaged to the TSC complex are needed to both validate these models and provide new insights into regulatory functions of the TSC complex.

mTORC1 signaling in human disease

Germline or loss of function mutations on TSC1 and TSC2 leads to aberrant mTORC1 activation observed in lesions of the genetic tumor syndrome tuberous sclerosis complex (TSC). TSC affects up to one in 6,000 births and is characterized by the formation of benign tumors in multiple organ systems including skin, brain, heart, kidneys and other organs. Clinical

manifestations can vary widely with a diversity of pathophysiological phenotypes including malformations of the cerebral cortex, cardiac rhabdomyomas, renal angiomyolipomas, facial angiofibromas, and many neurological pathologies such as epilepsy, autism spectrum disorder, learning difficulties and behavior problems[134]. Loss of TSC complex function through direct mutations on TSC1 and TSC2 can also cause pulmonary lymphangioleiomyomatosis (LAM), a destructive lung disease that exclusively affects women[135]. Moreover, genetic evidence has also indicated tumor specific loss of function of TSC1 and TSC2 in sporadic cancers such as bladder and liver cancer[136,137]. Both cell lines and patient-derived xenografts of hepatocellular carcinomas (HCCs) contain mutations of both TSC1 and TSC2, while up to 13% of bladder cancers contain TSC1 mutations[137-139]. Of note, the homozygous loss of TSC1 or TSC2 in humans has yet to be reported and knockout of these genes in mice results in early embryonic lethality[140-142]—highlighting the essential role of the TSC complex in regulating mTORC1 activity.

Despite its role as the third core subunit of the TSC complex, loss-of-function mutations in the TBC1D7 gene have yet to be identified in TSC patients[35]. Rather, germline homozygous truncating mutations in TBC1D7 lead to its complete loss from the cell, causing a rare autosomal-recessive megalocephaly and neurocognition syndrome referred to as “TBC1D7 Syndrome”. Like TSC patients, individuals with this syndrome display a range of neuropsychiatric anomalies and intellectual disabilities, however, many of the other common clinical manifestations of TSC, like tubers and benign tumors have not been reported for these individuals[143,144]. Growth compatibility upon complete loss of TBC1D7 further differentiates this third, non-essential, unit of the TSC complex from TSC1 and TSC2. Given the rarity of patients with TBC1D7 Syndrome, a complete clinical characterization, and the molecular underpinnings

driving this condition, is limiting. However, a recent report from our lab characterizing a mouse knockout model of TBC1D7 has highlighted a critical role of mTORC1 signaling in driving brain overgrowth, resulting in a megalencephaly phenotype, upon loss of TBC1D7[145].

In parallel to TSC complex mutations, germline and loss-function mutations in all three of the genes (DEPDC5, NPRL2 & NPRL3) that comprise GATOR1 have been identified in patients with focal epilepsies associated with malformations of cortical development (MCD). These patients present with a range of neurological pathophysiologies including seizures, intellectual disability, language delay, anxiety, attention-deficit disorder, and autism spectrum disorder[146]. Related to GATOR1 mutations are those identified in genes encoding components of the KICKSTOR protein complex, specifically KPTN and SZT2. Like GATOR1, patients with KICKSTOR mutations display neurological manifestations such as epilepsy, macrocephaly, neurodevelopmental delays, and intellectual disabilities[147-150].

Beyond the loss of function mutations found in the essential negative regulators of mTORC1, numerous oncogenic mutations are present in the pathways that directly regulate the TSC complex. As such, these mutations are major drivers of growth factor-independent activation of mTORC1 observed in most human cancers[136]. For example, within the PI3K-AKT pathway, loss of the tumor suppressor PTEN, the most important negative regulator of Akt through its dephosphorylation of PIP3 to PIP2, is observed in many human cancers and leads to constitutive activation of the pathway[151]. Additionally, activating mutations are found in the genes encoding both Akt and PI3K, the latter being one of the most ubiquitously activated oncogenes in all human cancers[152,153]. Within the RAS-Erk pathway, oncogenic mutation of both Ras and BRAF promote hyperactive signaling observed in over 85% of cancers[154]. Finally,

growth-factor independent hyperactivation of both signaling cascades is promoted through oncogenic mutation of RTKs and oncogenic fusion proteins[136,155].

Overview of the dissertation

In the past 20 years, remarkable progress has been made towards understanding the highly intricate signaling network upstream of mTORC1. However, as highlighted in this chapter, many of the mechanistic details of the regulatory proteins in this network, specifically the TSC complex and Rheb, have yet to be fully described. An observation that the TSC complex preferentially binds to Rheb in its inactive form led me to hypothesize that the TSC complex contains unrecognized regulatory functions towards Rheb outside of its canonical GAP activity. In this dissertation, I have investigated this novel binding interaction between the TSC complex and Rheb.

In chapter two, I first validate a prior discovery that the TSC complex preferentially binds to Rheb in its GDP-bound form and confirm that this binding is a real biochemical property of the TSC complex. I then expand on these findings and show that the interaction is direct, highly conserved, and occurs outside of the canonical GAP domain of TSC2. Finally, I employ Cross Linking Mass Spectrometry (CLMS) to broadly map putative interaction domains between Rheb-GDP and TSC2. I find that crosslinks cluster on divergent loop regions of TSC2 and in the absence of these loops, the TSC complex loses its preferential binding to Rheb-GDP. Importantly, I demonstrate that the loop regions of TSC2 are important for suppression of mTORC1 signaling upon growth factor withdrawal from the cell.

In chapter three, I first demonstrate that phosphorylation on TSC2 diminishes the binding interaction between the TSC complex and Rheb-GDP. In follow up to this, I provide an updated

hypothetical model of TSC complex lysosomal localization that requires the loop regions. I also provide perspectives on how these loops may be important for Rheb regulation and extend this to an overview of the conservation of these loop regions and their broader role in mTORC1 signal integration. In conclusion, I discuss the immediate and long-term future directions that are necessary to round out the findings made in this dissertation.

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Chapter 2:

A GAP-independent binding interaction between the TSC complex and Rheb reveals dual modes of mTORC1 regulation by the TSC complex

Alphafold protein structure modeling for figure 2.8 and 2.9 were done by Kenny Wang

Radio-labelled nucleotide loading experiments for figure 2.1B-C were done by Aaron Hosios

ABSTRACT

The mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) is a master signaling node that integrates signals in the form of nutrients, growth factors, cytokines and hormones to coordinate metabolic processes underlying cell growth. mTORC1 is under the direct control of two systems of GTPases, the Rag GTPase heterodimer and Rheb. In nutrient replete conditions, the Rag GTPases recruit mTORC1 to the surface of the lysosome where it is directly activated by a lysosomal pool of Rheb. The activation state of Rheb is tightly controlled by the tuberous sclerosis complex (TSC), a large protein complex comprised of the tumor suppressors TSC1 and TSC2 and a third protein, TBC1D7. Through a small domain at the C-terminus of TSC2, the TSC complex acts as a GTPase Activating Protein (GAP) to promote active, GTP-bound Rheb to inactive GDP-bound Rheb, whereby it can no longer activate mTORC1. Major growth factor signaling pathways converge on the TSC complex to inhibit its ability suppress mTORC1 signaling, primarily through inhibitory phosphorylation events on TSC2 that promote dissociation of the TSC complex from the lysosome, where mTORC1 activation by Rheb occurs. Despite significant progress in understanding these spatial regulation events, many longstanding questions underlying the mechanistic function of the TSC complex, and its regulation of Rheb remain unanswered. Here, we report that the TSC complex preferentially engages Rheb in is inactive, GDP-bound form and that this interaction is required for complete mTORC1 suppression upon growth factor withdrawal from the cell. Our results reveal that the preferential binding occurs through divergent and highly phosphorylated loop regions on TSC2, that have evolved to provide a second layer of regulation over Rheb-mTORC1 signaling.

INTRODUCTION

Cells sense and respond to growth signals to alter metabolic processes accordingly. Anabolic and catabolic processes underlying regulated growth are controlled by a ubiquitous signaling network that receives signals from nutrients, growth factors, cytokines and hormones. The pathways sensing these signals converge on the protein kinase complex mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) which functions to integrate these diverse cues to coordinate metabolic changes that underlie cell growth[1,2].

The ability of growth factor signaling pathways to activate mTORC1 is tightly controlled by a protein complex comprised of the tuberous sclerosis complex (TSC) tumor suppressors, TSC1 and TSC2, and the protein TBC1D7. The TSC complex functions as a direct and essential negative regulator of mTORC1 through its GTPase activating protein (GAP) activity towards the small G-protein, Ras homolog enriched in brain (Rheb)[3-5]. Contrary to its name, Rheb is ubiquitously expressed and is essential for mTORC1 activation. Only when Rheb is GTP-bound can it bind and activate mTORC1[6,7]. Thus, the TSC complex acts as a potent inhibitor of mTORC1 activation, at least in part, by stimulating the intrinsic GTPase activity of Rheb, promoting its conversion to Rheb-GDP, which cannot activate mTORC1[4].

mTORC1 activation occurs on the cytosolic face of lysosomes, where it can integrate signals from both amino acids and growth factor signaling pathways**.** Amino acid inputs promote mTORC1 activation by stimulating its docking at the lysosome through interactions with the Rag family of small GTPases[4,8-11]. Unlike Rheb, Rag protein binding to mTORC1 does not activate it. However, the recruitment of mTORC1 to the lysosome serves to bring it into proximity to its direct activator Rheb, a sub population of which resides at the lysosome[8,12]. Thus, the activation

state of mTORC1 is ultimately decided by whether or not lysosomal Rheb is bound to GTP or GDP, which is regulated by the TSC complex.

While many other GTPases have established known regulators such as Guanine Nucleotide Exchange Factors (GEFs) and Guanine Dissociation Inhibitors (GDIs)[13], the TSC complex remains as the only known and established direct regulator of Rheb. Despite the size of the TSC complex (upwards of 700 kDa)[14], its regulatory domain is mapped exclusively to the C-terminus of TSC2 where the GAP domain resides[4,15,16]. Here, we describe a novel interaction between the TSC complex and Rheb. We find that the TSC complex preferentially engages Rheb in its inactive, GDP-bound form, through a domain outside of the established GAP domain. Furthermore, we determined that unstructured regions on TSC2, which we refer to as TSC2 loops, mediate this unique and preferential binding to Rheb-GDP. Our data suggests that the TSC complex has two distinct binding modes: the canonical GAP mode that engages Rheb-GTP to stimulate GTP hydrolysis, and a second mode, which we refer to as the "association mode" that strongly engages Rheb-GDP to provide a second layer of mTORC1 regulation at the lysosome.

RESULTS

The TSC complex preferentially binds to GDP-bound Rheb

We previously reported that the TSC complex localizes to the lysosome in the absence of growth factors, and that this localization is dependent on farnesylated Rheb[7]. To investigate the molecular nature of the interaction between the TSC complex and Rheb, the ability of endogenous TSC complexes in cell lysates to bind to recombinant GST-Rheb loaded with GTPγS or GDP was determined. Intriguingly, the TSC complex exhibited a strong preference for binding to Rheb-GDP relative Rheb-GTPγS (Fig. 2.1A). This preferential interaction between

Figure 2.1. The TSC complex preferentially binds to Rheb in its inactive, GDP-bound form. **(A)** Endogenous TSC complex components were pulled down from lysates of serum-starved (16 hours) 293E cells with recombinant bacterially purified GST-Rheb, or the control protein Rab5A, preloaded with no nucleotide (none), GTPγS (500 μm), or GDP (500 μm). GST-fused bait proteins were detected with Ponceau S stain. **(B)** Recombinant purified GST-Rheb (0.2 μm) was loaded with radio-labelled α-32-P-GDP (5 nM) or α-32-P-GTP (5 nM) for up to two hours on ice, passed through a nitrocellulose filter, dried and the amount of nucleotide bound to Rheb was exposed by phosphorimager. **(C)** Rheb was loaded as in (B) for two hours with increasing concentrations of cold corresponding nucleotide as indicated on the figure. **(D)** Endogenous TSC complex components were pulled down as in (A) with GST-Rheb preloaded with no nucleotide (none) or a range of GTPγS or GDP concentrations (10 μm to 1 mM) and compared to GST-Rab5A loaded with the highest concentration of GDP (1 mM). **(E)** TSC complex components were pulled down as in (A) with GST-Rheb or the control protein, GST-Rab5A, preloaded with no nucleotide (none) or a panel of nucleotide analogs as indicated on the figure. **(F)** TSC complex components pulled down as in (A) with a panel of recombinant purified GST-tagged GTPases preloaded with nucleotide as in (A).

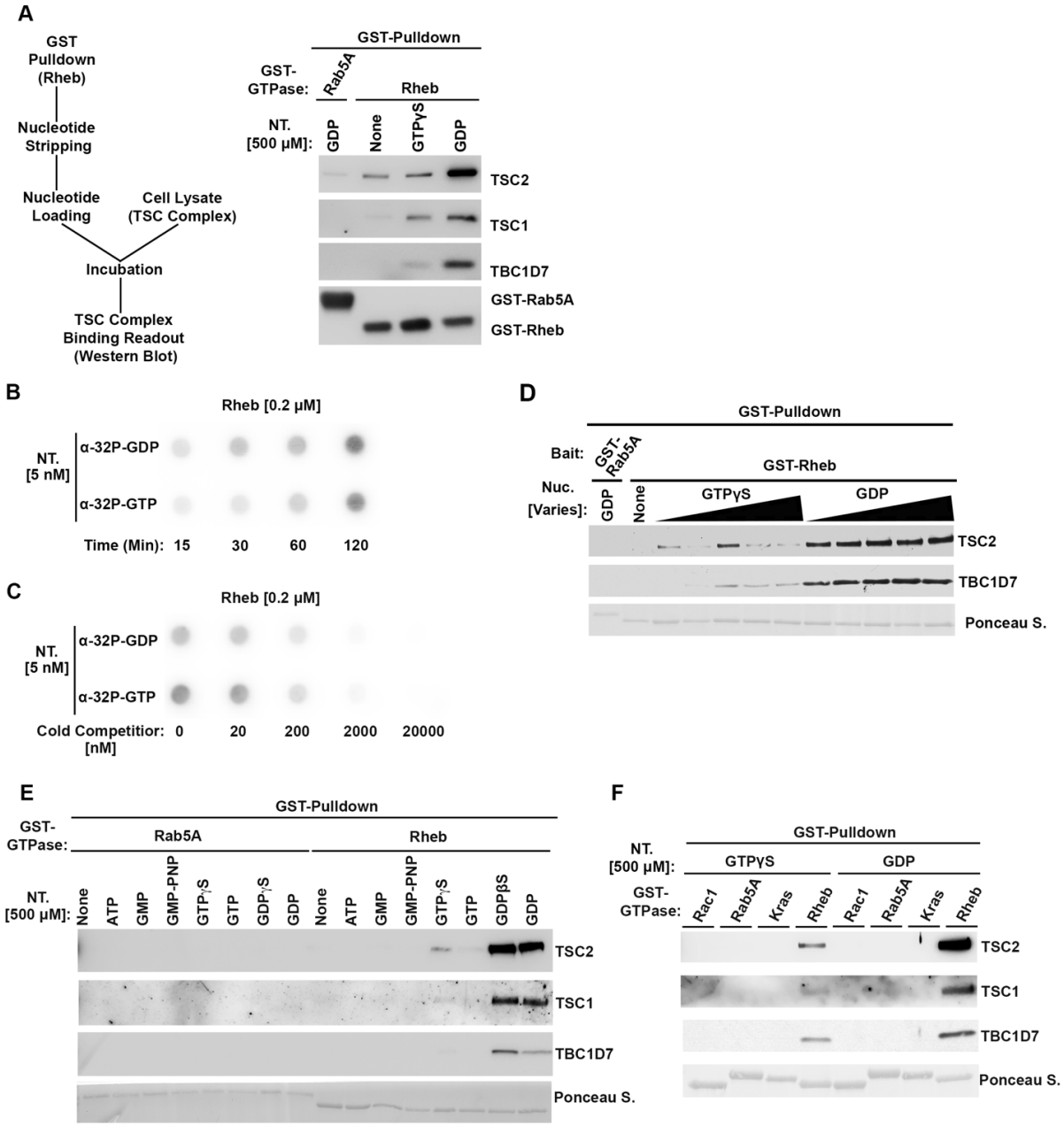


Figure 2.1 (Continued).

Rheb-GDP and the TSC complex was not due to differences in guanylate binding to Rheb, as both loading (Fig. 2.1B) and release (Fig. 2.1C) kinetics of Rheb for GDP and GTP were similar. Furthermore, the TSC complex preferentially bound to Rheb-GDP loaded over a range of guanylate concentrations (10 μm – 1 mM), even at a 100-fold lower concentration of GDP versus GTP (Fig. 2.1D). To further characterize the importance of the purine nucleotide in the binding of Rheb to the TSC complex, Rheb was loaded with a panel of nucleotides, including ATP, GTP, GTP analogs (GMP-PNP and GTPγS), GDP, and a GDP analog (GDPβS). Again, the TSC complex bound with a strong preference to Rheb-GDP and Rheb-GDPβS (Fig. 2.1E). Importantly, the TSC complex did not show any binding above background to GST-Rab5A loaded with these nucleotides. The binding preference of the TSC complex for GDP-bound GTPases was specific to Rheb, as we detected no TSC complex components in pulldowns of GTPγS- or GDP-loaded GST-Rac1, Rab5A, or K-Ras, whereas the TSC complex robustly bound Rheb-GDP and to a much lesser extent Rheb-GTPγS (Fig. 2.1F).

Farnesylation status, a post-translational C-terminal modification on Rheb, also did not influence the preferential binding between the TSC complex and Rheb-GDP. Rheb purified from bacteria is in its unfarnesylated form, while Rheb purified from mammalian cells is farnesylated[17]. Repeating the pull-downs in a side-by-side comparison of GST-Rheb purified from bacterial or mammalian cells revealed the same preferential binding pattern between the TSC complex and Rheb-GDP, regardless of the host purification system (Fig. 2.2A). Consistent with this result, when GST-Rheb was purified from mammalian cells following overnight treatment with a farnesyl transferase inhibitor (FTI), no difference in binding preference was observed between the FTI treated or untreated purified Rheb proteins (Fig. 2.2B). Moreover, purified wild-type Flag-Rheb and the farnesylation deficient mutant of Rheb (Flag-

RhebΔCAAX) both preferentially engaged the TSC complex when GDP-bound (Fig 2.2C). Curiously, we noticed a slight but reproducible increase in binding between Rheb-GDP and the TSC complex in all conditions in which Rheb was unfarnesylated compared to its farnesylated control. These observations suggest that Rheb farnesylation status does not dictate the preferential binding between Rheb-GDP and the TSC complex, however a lack of farnesylation on Rheb may enhance the binding interaction. .

Figure 2.2. Farnesylation status does not change the preferential binding of the TSC complex for Rheb-GDP. (A) Endogenous TSC complex components were pulled down from lysates of serum-starved 293E cells with GST-Rheb that had been purified from either bacterial cells or mammalian 293E cells and compared to the control peptide, GST, which had also been purified from either bacterial or mammalian cells. GST-Rheb or GST was preloaded with no nucleotide (none), GTPγS (500 μm), or GDP (500 μm). GST-fused bait proteins were detected with Ponceau S stain. **(B)** Endogenous TSC complex components were pulled down as in (A) with GST-Rheb purified from 293E cells that had been pretreated for 16 hours prior to protein purification with or without a farnesyltransferase inhibitor (10 μm) prior to cell lysis. Pre-loading of Rheb with nucleotide was done as in (A). **(C)** Endogenous TSC complex components were pulled down as in (A) with flag-tagged wild-type Rheb or the farnesylation defective mutant, Rheb C181S (ΔCAAX mutant), purified from 293E cells and compared to control peptide, Flag, also purified from 293E cells. Preloading of Rheb with nucleotide was done as in (A).

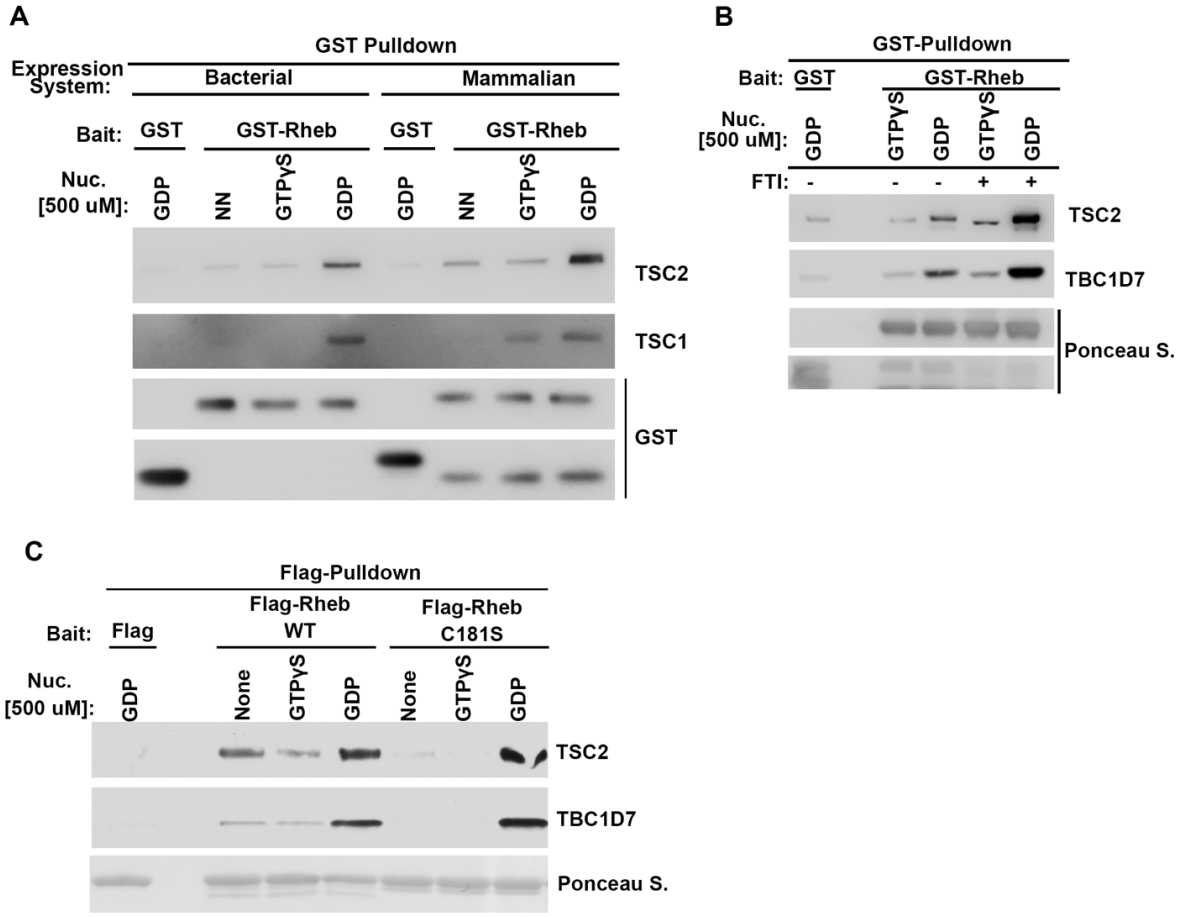


Figure 2.2 (Continued).

The TSC complex binds to Rheb-GDP through TSC2

In the cell, TSC2 exists both as a subunit of the TSC complex and on its own in a "free pool"[7]. To establish whether TSC2 could preferentially bind to Rheb-GDP without being fully engaged in the TSC complex, TSC2 was exogenously expressed on its own or in combination with the other two TSC complex components (TSC1 and TBC1D7). Interestingly, TSC2 lost its ability to bind Rheb loaded with either guanylate when it was expressed on its own. However, addition of TSC1 into the system restored the strong interaction between Rheb-GDP and TSC2. TBC1D7 was dispensable for the interaction as no additional binding was observed upon introduction of TBC1D7 into the system (Fig. 2.3A).

Ruling out the possibility that the TSC complex binds to Rheb-GDP through TSC1, when TSC1 and TBC1D7 were fished out of $TSC2^{-/-}$ mouse embryonic fibroblasts (MEF) cell lysates, no binding to Rheb was observed. As expected, all TSC complex subunits fished out of $TSC2^{+/+}$ MEF cell lysates preferentially bound to Rheb-GDP (Fig. 2.3B). This indicated that the TSC complex binds to Rheb through TSC2, but requires TSC1 for stabilization of the interaction. We employed a parallel genetic system, $TSC1^{-/-}$ MEFs, to validate the finding that the TSC1-TSC2 interaction is required for the interaction between Rheb-GDP and TSC2. As expected, all TSC complex components preferentially bound to Rheb-GDP in the $TSC1^{+/+}$ MEF setting. In the $TSC1^{-/-}$ MEF setting, TSC2 and TBC1D7 lost their preference for Rheb-GDP, supporting the initial finding that free TSC2 cannot bind to Rheb-GDP (Fig. 2.3C).

Figure 2.3 The TSC complex binds to Rheb-GDP through TSC2 and full complex assembly is required for the interaction. (A) Exogenously expressed TSC complex components were fished out of 293E cell lysates containing 1) individually expressed Flag-TSC2 2) co-expression of Flag-TSC2 with Myc-TSC1 or 3) co-expression of Flag-TSC2, Myc-TSC1 and HA-TBC1D7 with GST-Rheb, or the control protein GST-Rab5A, preloaded with no nucleotide (none), GTPγS (500 μm), or GDP (500 μm) **(B)** Endogenous TSC complex components were pulled down as in (A) from $TSC2^{+/+}$ or $TSC2^{-/-}$ MEFs with GST-Rheb, or the control GST-Rab5A, preloaded as in (A). **(C)** Endogenous TSC complex components were pulled down as in (A) from $TSC1^{+/+}$ or $TSC1^{-/-}$ MEFs with GST-Rheb, or the control GST-Rab5A, preloaded as in (A). **(D)** HeLa cells were serum starved overnight (16 hours) prior to cell lysis and fractionation by SEC. The fractions containing the TSC complex, the free pool of TSC2 and the free pool of TBC1D7 were collected and pooled. Endogenous TSC complex components were fished out of the separate pooled fractions with GST-Rheb, or the control protein GST-Kras, preloaded with no nucleotide (none), GTPγS (500 μm), or GDP (500 μm).

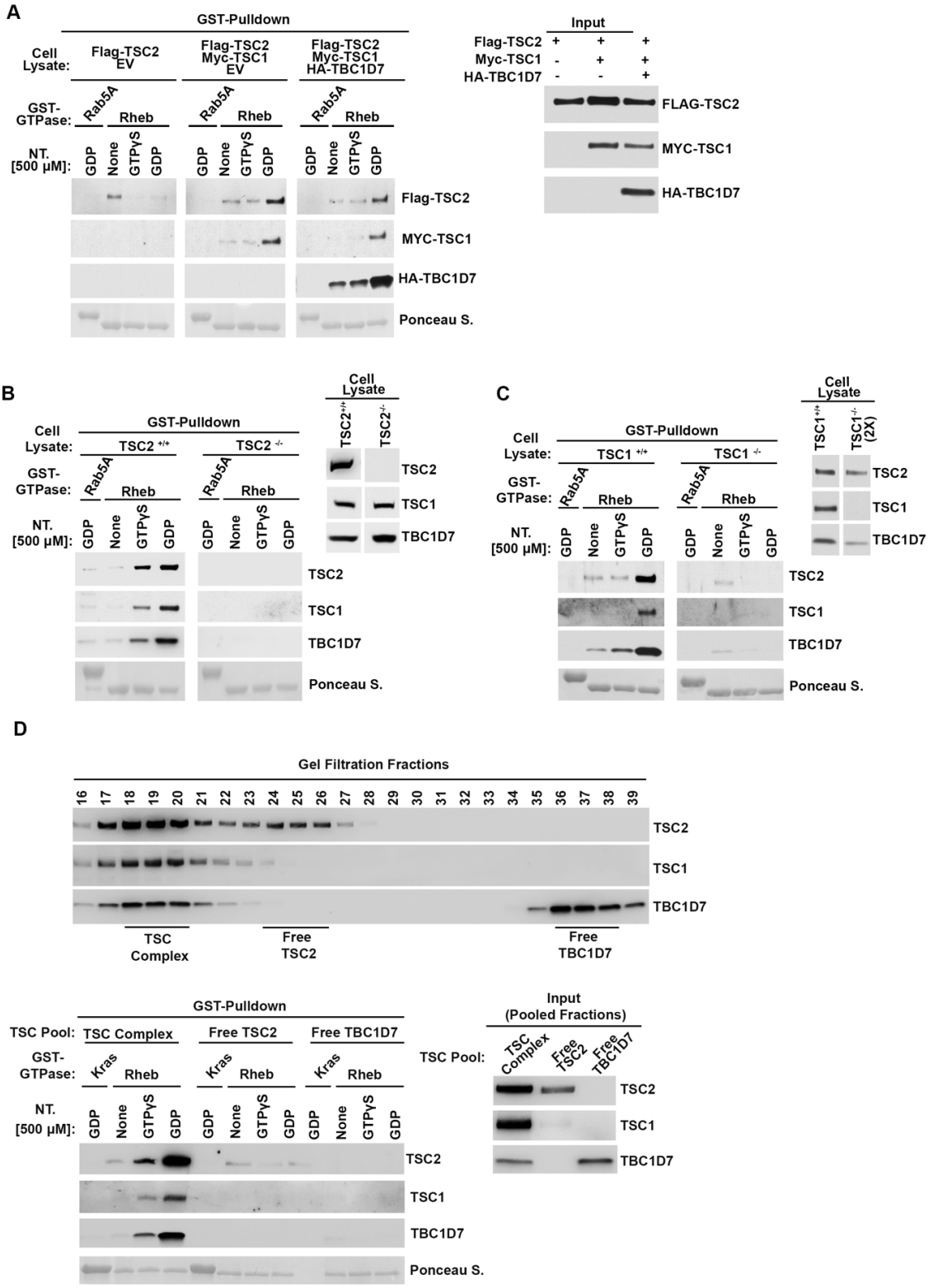


Figure 2.3 (Continued).

As previously described, two separate pools of TSC2 exists in cells. By size exclusion chromatography (SEC), the TSC complex fractionates around 2 MDa while the free pool of TSC2 fractionates around 600 kDa. Free TSC1 does not exist outside of the full TSC complex, while a free pool of TBC1D7 exists outside of the complex and fractionates around 34 kDa[7]. To understand how the disparate TSC2 pools from the same cell lysate behave in the context of Rheb binding, we separated the free pool of both TSC2 and TBC1D7 from the TSC complex by SEC. In line with the overexpression and genetic evidence that TSC2 requires TSC1 to bind to Rheb-GDP, we only observed binding of TSC2 to Rheb-GDP from the SEC fractions containing the TSC complex. The free pool of TSC2 from the same cell lysate lost its ability to strongly engage Rheb in either loaded state. No binding of the free pool of TBC1D7 to Rheb was detected (Fig. 2.3D). Collectively, these findings reveal that the strong binding between TSC2 and Rheb-GDP requires the TSC2-TSC1 interaction, supporting a role for TSC1 whereby it positions TSC2 in a confirmation that enables it to engage nucleotide-bound Rheb.

The preferential binding of the TSC complex for Rheb-GDP is conserved in *S. pombe*.

The fission yeast, *Schizosaccharomyces pombe* (*S. pombe*) contains homologs of both TSC1 and TSC2 (a homolog for TBC1D7 has yet to be identified), and the Rheb homolog, Rhb1[18,19]. *S. pombe* TSC2 is highly conserved in the N-terminal HEAT repeats and GAP domain but lacks most of the unstructured/dimer domain present in the middle of mammalian TSC2 (Fig. 2.4A)[20]. We sought to characterize the conservation of the interaction between Rheb-GDP and the TSC complex in this system to further elucidate the functional significance of the interaction and narrow down the interaction domain on TSC2.

Figure 2.4. The preferential binding of the TSC complex for Rheb-GDP is conserved in *S. pombe*. **(A)** Human and *S. pombe* TSC2 primary structure with conserved domains indicated in light pink and non-conserved regions indicated in red. **(B)** Schematic of *S. pombe* epitope-tagged TSC1 (HA) /TSC2 (Flag) cell line in which gene cassettes were inserted into the endogenous loci of TSC1 and TSC2. **(C)** Flag-Immunopurification (TSC2 handle) from the TSC1/TSC2 tagged *S. pombe* cell lysate compared to a control immunopurification with agarose beads. **(D)** Immunoblots of SEC fractions from TSC1/TSC2 tagged *S. pombe* cell lysate. **(E)** SEC fractions from (D) containing the two different TSC complex pools and the free pool of TSC2 were collected and pooled and TSC complex components were fished out of the separate pooled fractions with GST-Rhb1, or the control protein GST-Ras1, preloaded with GTPγS (500 μm), or GDP (500 μm).

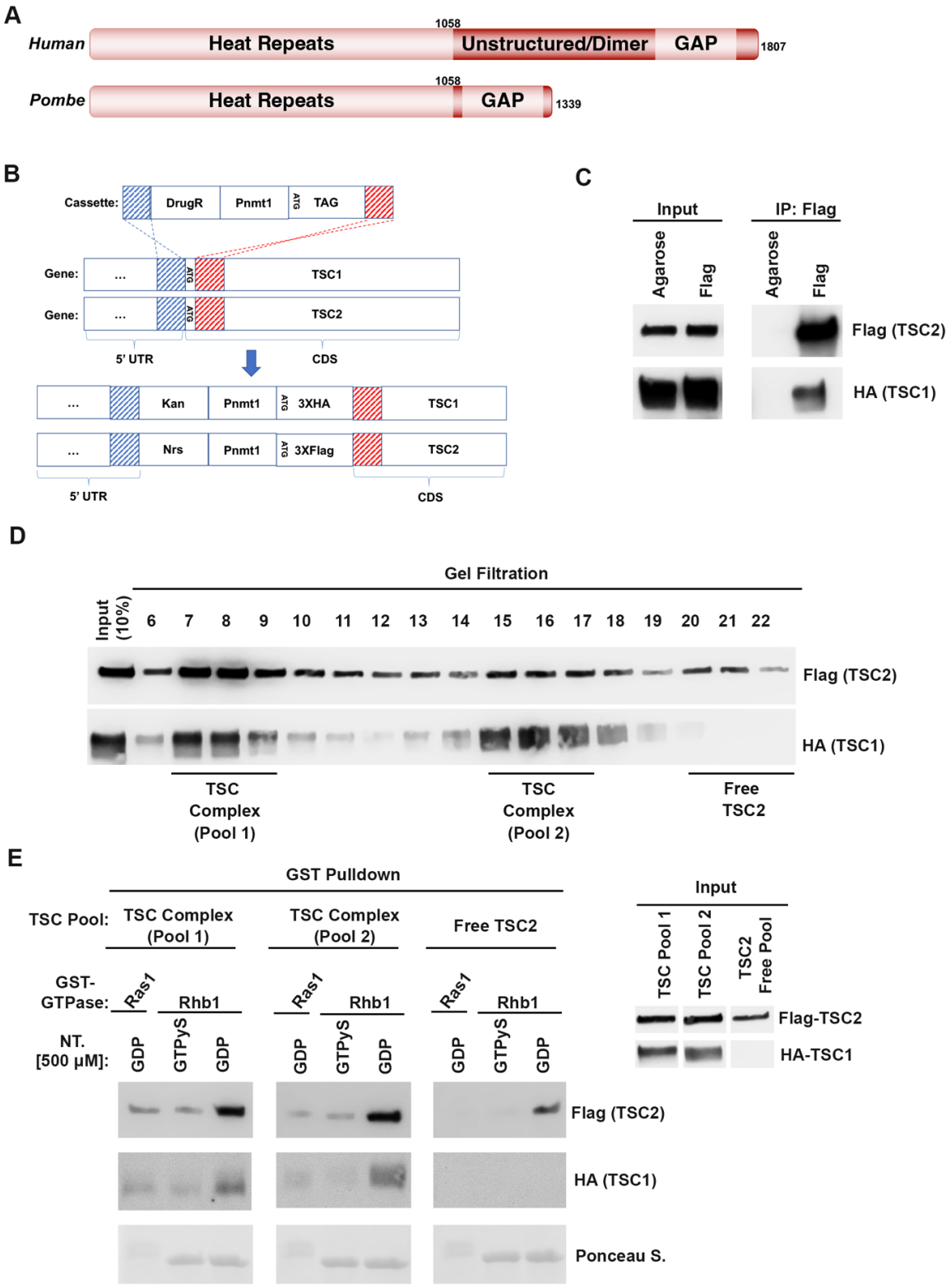


Figure 2.4 (Continued).

To study this, we generated a new *S. pombe* cell line that co-expresses tagged versions of endogenous TSC1 (HA) and TSC2 (Flag) under an exogenous promoter (Fig. 2.4B). We confirmed that TSC1 co-immunopurified with TSC2, validating an intact TSC complex in the new *S. pombe* strain (Fig 2.4C). The cell line was fractionated by SEC, and we detected two disparate high molecular weight pools containing the TSC complex and a third lower molecular weight pool containing free TSC2 (Fig. 2.4D). This contrasted with the two pools of TSC2 (complex and free TSC2) detected in the mammalian HeLa cell line (Fig. 2.3D). Based on SEC studies in the mammalian system with overexpressed TSC complex components (data not shown), we believe that TSC complex pool 1 (Fractions 7-9, Figure 2.4D) is driven by overexpression of TSC1 and TSC2, and likely does not exist at endogenous levels. TSC complex pool 2 (Fractions 15-17, Figure 2.4D) elutes in the fractions directly following the fractions where endogenous mammalian TSC complex elutes (data not shown, the FPLC columns used in figure 2.3D and 2.4D are not the same), tracking with the reduced size of *S. pombe* TSC1 (103 kDa) and TSC2 (154 kDa) compared to mammalian TSC1 (130 kDa) and TSC2 (200)[18,21,22]. This second TSC complex pool (pool 2) likely reflects the biologically relevant TSC complex in *S. pombe*.

When we fished the TSC complex out of all three distinct SEC pools, we found that TSC2 preferentially engaged Rhb1-GDP when it was engaged in the complex (pool 1 and pool 2) and also when it was isolated on its own (pool 3), in contrast to the mammalian free pool of TSC2 (Fig 2.4E). This indicated that the preferential binding between the TSC complex and Rheb-GDP is a highly conserved interaction. The discrepancy between Rheb/Rhb1-GDP and free TSC2 in the human vs. *S. pombe* system is likely due to structural differences such that *S. pombe* TSC2 does not require TSC1 stabilization to bind Rhb1-GDP. Given the binding conservation,

this finding also suggested that the interaction interface may occur within a conserved region on TSC2.

Purified TSC complex preferentially binds to Rheb-GDP in a reconstituted system.

The conserved nature of the interaction led us to ask if the interaction was direct or required additional regulators to maintain the strong binding interaction between the TSC complex and Rheb-GDP. First, we established purifications strategies for both Rheb and the TSC complex to test the interaction in a reconstituted system. Rheb was purified from a bacterial system and the TSC complex was purified from 293 Freestyle mammalian cells. The TSC complex eluted in one large peak by SEC, as shown in the FPLC trace. Coomassie staining of both Rheb and the TSC complex demonstrates highly pure final products for subsequent binding studies (Fig. 2.5A).

Consistent with previous results, in a reconstituted system, the TSC complex preferentially engages Rheb-GDP, and the binding gradually increases as increasing amounts of Rheb-GDP are titrated into the system. Importantly, even at the highest concentration of Rheb-GTPγS the amount of TSC complex binding barely reached background levels (Fig. 2.5B). We repeated these studies with the GST epitope tag cleaved from Rheb and confirmed preferential binding of the TSC complex to Rheb-GDP over Rheb-GTPγS (Fig. 2.5C). The binding interaction between the TSC complex and Rheb-GDP became increasingly stronger as increasing concentrations of Rheb-GDP were added into the system (Fig. 2.5D).

Figure 2.5. Purified TSC complex preferentially binds to Rheb-GDP in a reconstituted system. **(A)** FPLC trace of the TSC complex, with the TSC complex peak indicated and Coomassie staining of purified Rheb and the TSC complex subunits indicated. **(B)** Purified TSC complex (5 nM) was incubated with increasing amounts of purified GST-Rheb (5 – 50 nM), or the control protein GST-Kras (50 nM), preloaded with GTPγS (500 μm), or GDP (500 μm) for 1 hour on ice. Incubated proteins were Flag-immunopurified (30 minutes) to detect the amount of Rheb bound to the TSC complex **(C)** Purified TSC complex (5 nM) was incubated with GST-free Rheb (100 nM) preloaded with GTPγS or GDP as in (B) and subsequently immunopurified as in (B). **(D)** Purified TSC complex (5 nM) was incubated with increasing amounts of purified GST-free Rheb (5 – 200 nM) preloaded with GDP (500 μm) and subsequently immunopurified as in (B). **(E)** Purified TSC complex (50 nM) was incubated on ice for 1 hour with with Rheb (1 uM) that had been preloaded with GDP (500 μm). The crosslinker DSP [10 uM] or DMSO was added to the incubation for an additional 30 minutes on ice and then quenched with Tris-HCL (50 mM). The incubations were subsequently fractioned by SEC and immunoblots are shown. **(F)** SEC fractions from (E) containing the TSC complex were collected and pooled (fractions 14-16). The TSC complex was subsequently Flag-immunopurified from the pooled fractions to detect the amount of Rheb bound to the TSC complex.

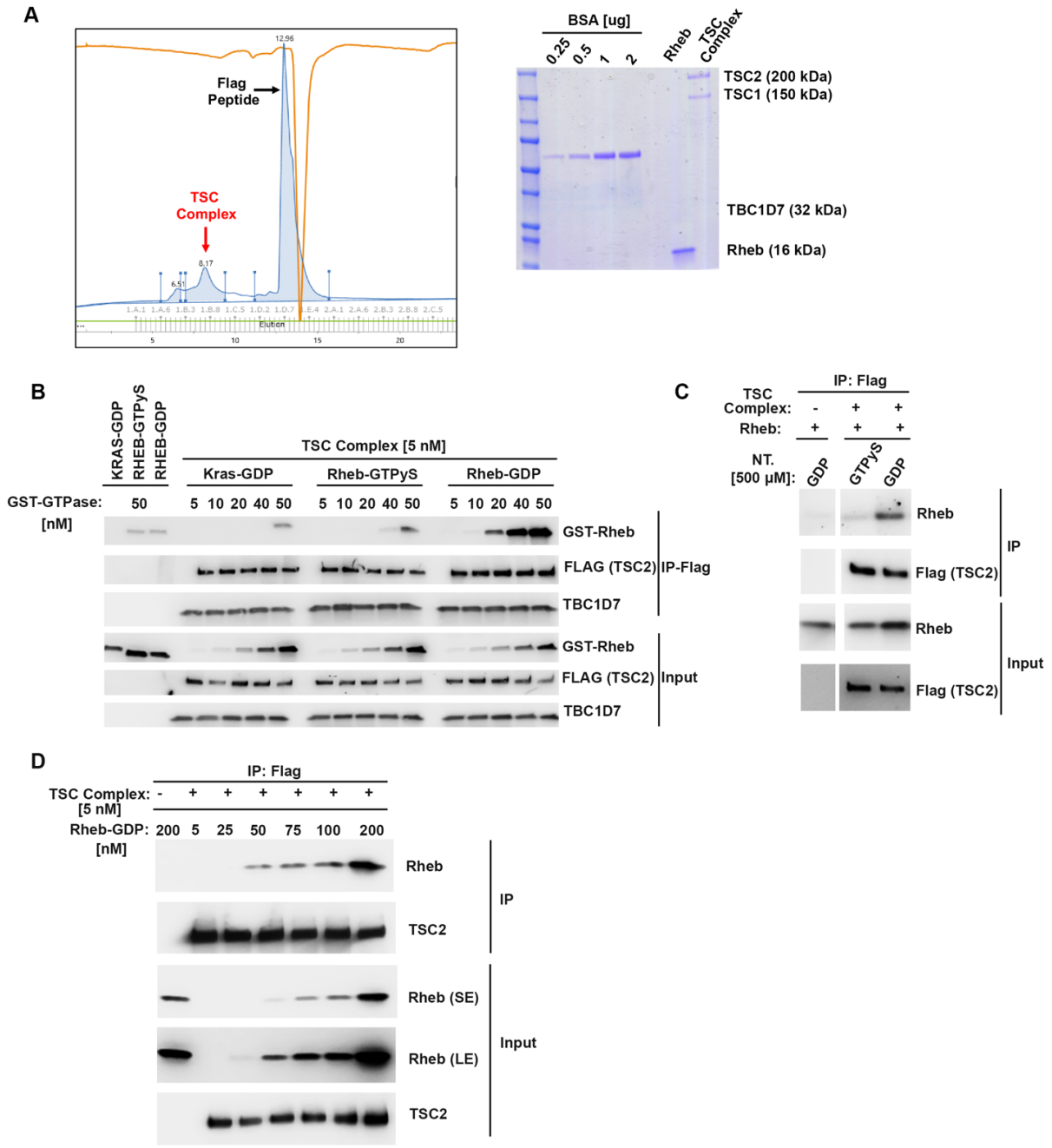


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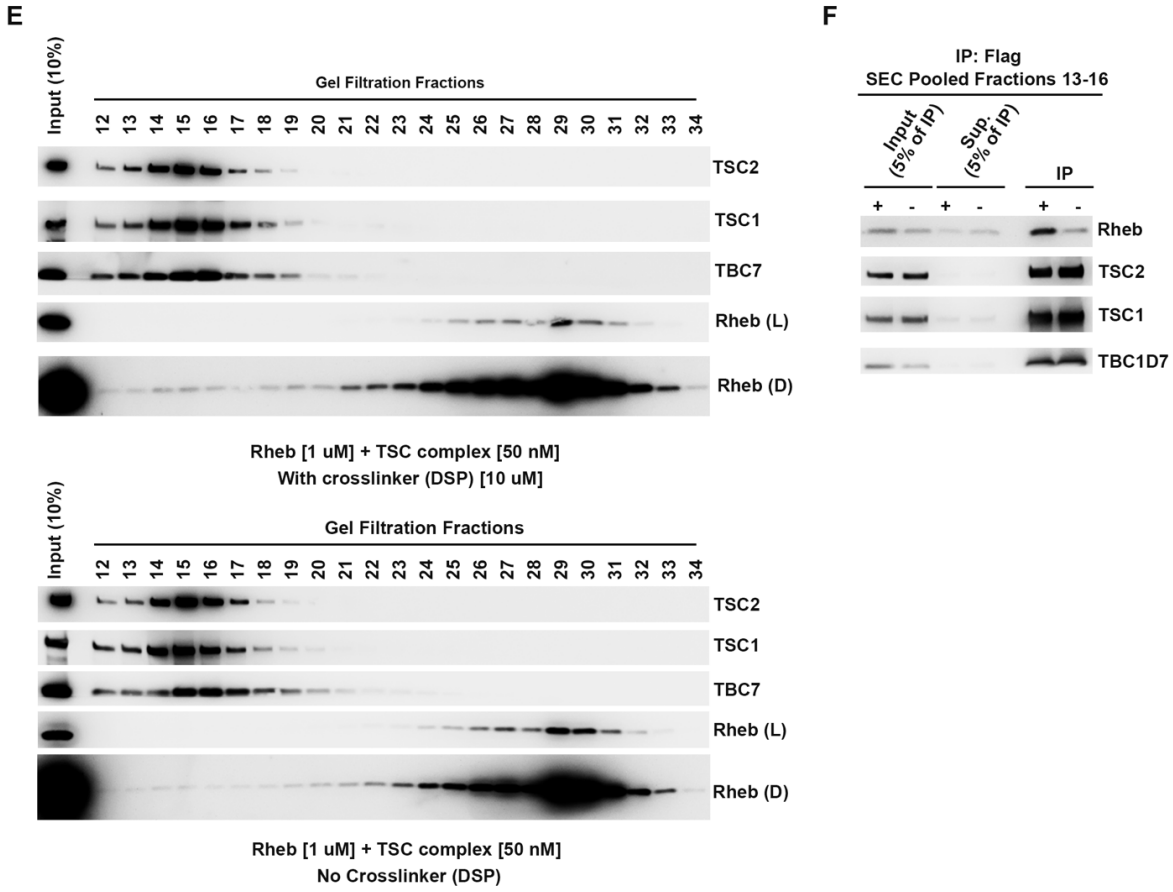


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To demonstrate the strength of this direct interaction, TSC complex/Rheb-GDP incubations were treated with or without a chemical crosslinker (DSP) and fractionated by SEC (Fig. 2.5E). Surprisingly, even in the absence of crosslinker, Rheb-bound TSC complexes were detected in the SEC pool containing the TSC complex (fractions 14 – 16). The crosslinked sample showed an increase in Rheb binding, informing us that crosslinker could be used to stabilize the interaction between Rheb-GDP and the TSC complex (Fig. 2.5F). Despite our detection of reconstituted Rheb-bound TSC complexes following SEC, a majority of the Rheb in both conditions does not engage the TSC complex, as evidenced by the lower molecular weight fractions in the SEC Rheb immunoblots (Fig. 2.5E). Despite adding a 20-fold molar excess of Rheb to TSC complex into the system, less than one percent of the Rheb appeared to co-fractionate with the TSC complex. The lack of complete engagement of Rheb in the system by the TSC complex is likely due to both constraints on the total number of Rheb-GDP binding sites in the system, as well as weak interactions that are not maintained during the SEC process.

The TSC complex preferentially binds to Rheb-GDP independent of the TSC2-GAP domain.

The sole established interaction interface between the TSC complex and Rheb is the catalytic GAP domain of TSC2, which stimulates the intrinsic GTPase activity of Rheb[4,15,16]. Several interacting residues between TSC2 GAP and Rheb in its active, GTP-bound form have been modeled. These interactions include both catalytic residues and residues that likely contribute to stabilization of the TSC2 GAP mode with Rheb-GTP[14,23]. We were curious if the interaction between the TSC complex and Rheb-GDP occurred within the highly conserved GAP domain[4,15,16], or in an alternative unknown Rheb-binding domain on TSC2.

TSC2 lacking the entire catalytic domain of TSC2 but containing the last ~80 residues directly following the GAP domain (TSC2ΔGAP) and a mutant lacking the entire GAP domain and the ~80 residues following it (TSC2ΔC) (Fig. 2.6A), form intact TSC complexes (Fig. 2.6B). These results were consistent with previous reports that the GAP domain of TSC2 is not required for TSC2-TSC1 interactions[23-26]. Not surprisingly, when we reconstituted HeLa TSC2 KO cells with the WILD-TYPEand GAPless mutants of TSC2, the GAPless mutants completely failed to suppress mTORC1 signaling upon growth factor withdrawal from cells (Fig. 2.6C). Strikingly, when we fished TSC complex components out of cells co-expressing TSC1, TBC1D7 and either the WILD-TYPEor either of the two GAPless mutants of TSC2, we observed preferential binding between Rheb-GDP and the TSC complex in all three overexpression conditions (WT, TSC2ΔGAP, and TSC2ΔC) (Fig. 2.6D). This exciting finding revealed that TSC2 binds to Rheb-GDP in a GAP-independent manner, suggesting that the TSC complex exhibits dual binding modes: a GAP mode with active, GTP-bound Rheb and a GAP independent-mode with inactive, GDP-bound Rheb.

Figure 2.6. The TSC complex preferentially binds to Rheb-GDP independent of the GAP domain on TSC2. (A) Primary structure of full-length WILD-TYPETSC2 and TSC2 truncation mutants lacking the GAP domain **(B)** TSC complexes from 293F cells expressing either Flag-TSC2 WT, Flag-TSC2 ΔGAP or Flag-TSC2 ΔC with co-expression of Myc-TSC1 and HA-TBC1D7 were pulled out of cell lysates by Flag-immunopurification and compared to an agarose-bead immunopurification control. **(C)** Immunoblot of HeLa TSC2 KO cells reconstituted with empty vector, Flag-TSC2 WT, Flag-TSC2 ΔGAP or Flag-TSC2 ΔC and serum starved for 16 hours prior to cell lysis. **(D)** TSC complexes prepared as in (B) were pulled out of cell lysates with GST-Rheb preloaded with GTPγS (500 μm), or GDP (500 μm) and compared to a control pull-down with GST-Kras preloaded with GDP (500 μm).

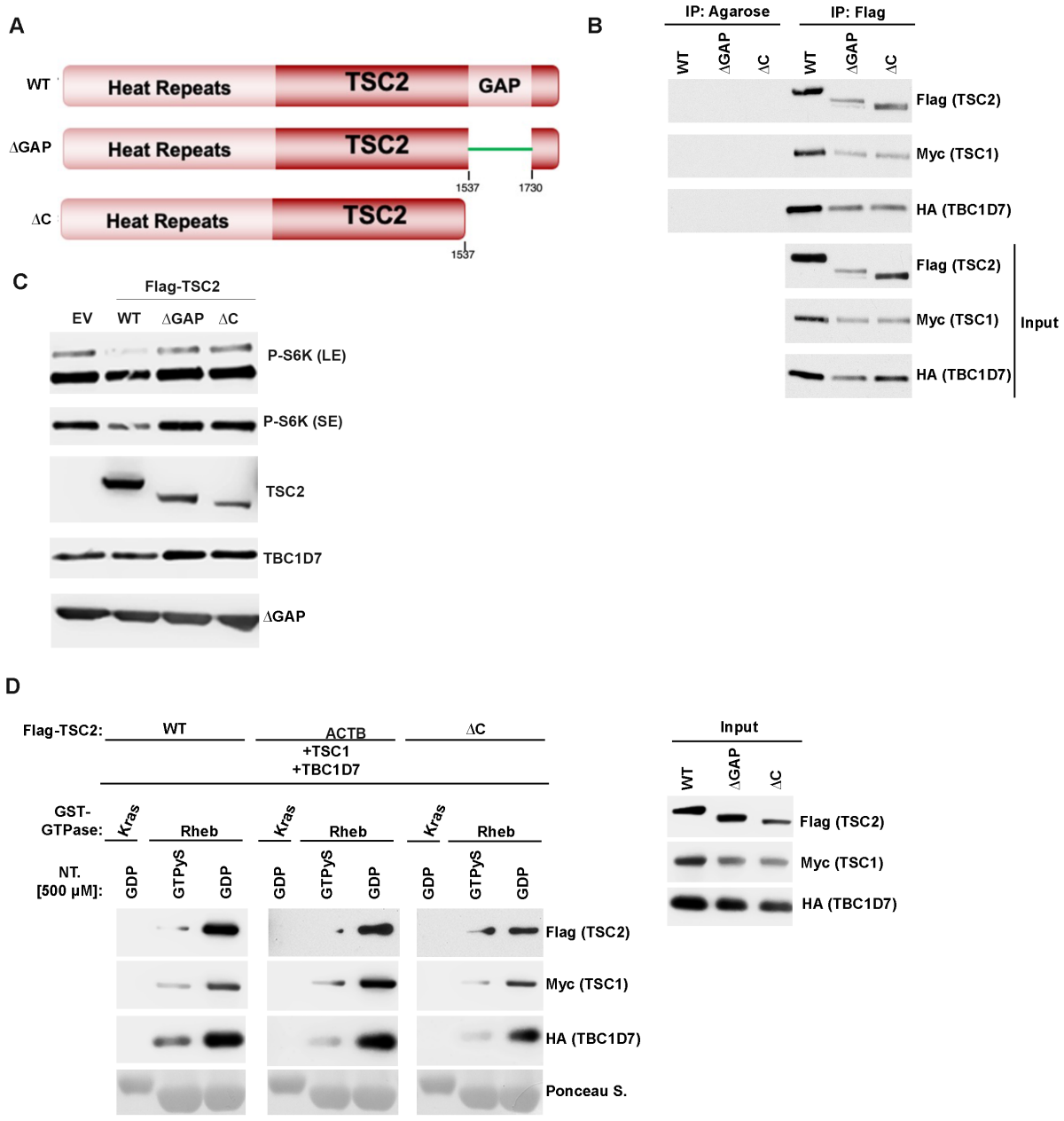


Figure 2.6 (Continued).

Extensive interactions between the TSC complex and Rheb-GDP through predicted unstructured regions on TSC2.

The important finding that TSC2 engages Rheb-GDP outside of the canonical GAP domain led us to next ask what alternative region of TSC2 was required for this strong and direct interaction. Recently resolved Cryo-EM structures of the TSC complex have revealed both the stoichiometry and detailed interconnectedness of TSC1, TSC2 and TBC1D7. The TSC complex is assembled in a 2:2:1 stoichiometry of TSC1, TSC2 and TBC1D7 with an asymmetrical organization. Two TSC2 molecules each form a seahorse-shaped conformation and interact through a dimerization domain located in the central region of TSC2, forming the "core" of the protein complex. In each TSC2 molecule, the GAP domain folds back on itself and makes intramolecular contacts with the N terminal heat repeats. The resolved Coil-Coil domain of the TSC1 molecules intertwine in parallel and make multiple contacts with the two TSC2 molecules, stabilizing the overall conformation of the complex. TBC1D7 is positioned at the C-terminal helices of TSC1 and does not make contact with TSC2[14].

Given the complexity and interconnectedness of the complex, coupled to the requirement of full complex assembly to capture the interaction between mammalian TSC2 and Rheb-GDP, we decided to use a crosslinking mass spectrometry (CLMS) approach to broadly map the region(s) of interaction in the context of the full TSC complex. The massive size differential between the TSC complex (700 kDa) and Rheb (21 kDa) prompted us to incubate highly pure TSC complex with 50-fold molar excess Rheb-GDP (Figure 2.7A) to enhance our likelihood of capturing novel interactions.

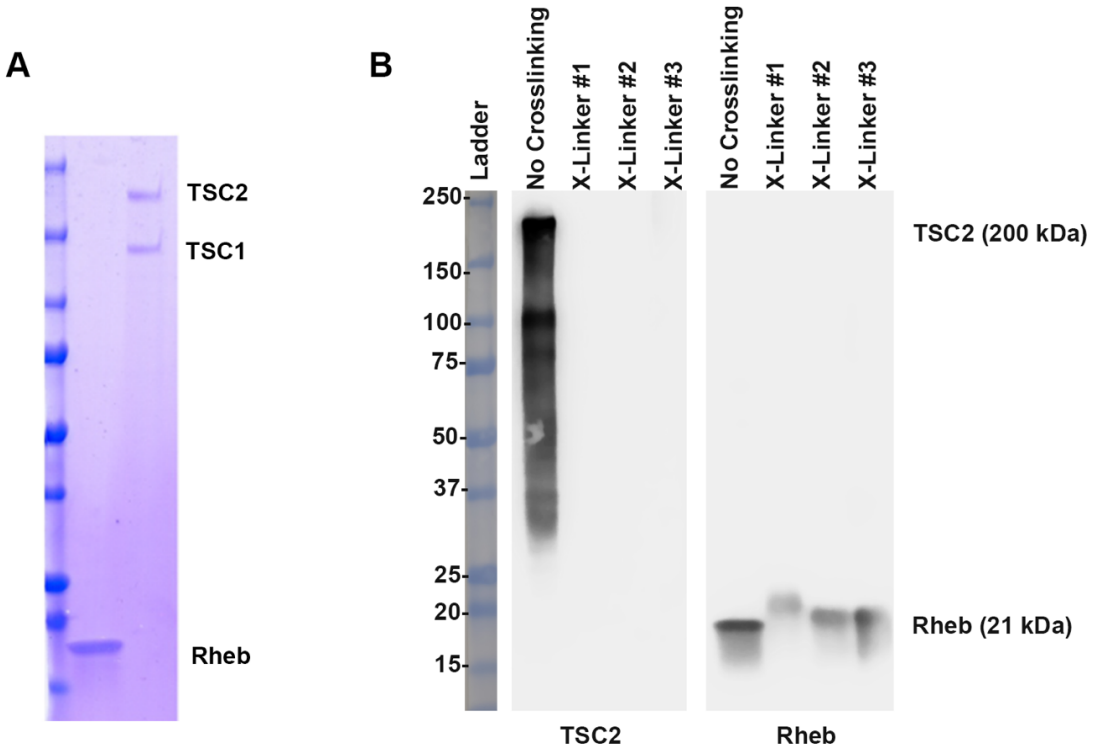


Figure 2.7. TSC complex and Rheb-GDP CLMS study validation. (A) Coomassie stained purified TSC complex and Rheb proteins submitted for CLMS. **(B)** TSC2 and Rheb immunoblots of the pre-crosslinking and crosslinked samples (BS3, EDC and E-EDC) that were submitted for CLMS. The samples were run on a non-reducing gel and compared to a non-crosslinked control sample.

Prior to analyzing our CLMS results, we ran aliquots of the incubated sample prior to crosslinking and after the reaction with the three different crosslinkers a non-reducing immunoblot. In the non-crosslinked sample, TSC2 appeared as a large smear and Rheb appeared as a dark band. In all three crosslinked samples, no TSC2 was observed on the immunoblot and lighter Rheb bands were detected. As non-reduced TSC complex is too large to enter the gel, and lighter Rheb indicated that at least a portion of the Rheb crosslinked to the complex, we validated that crosslinking occurred in the samples that were submitted for the CLMS study (Fig. 2.7B).

We first compared the top ten crosslinks, ranked by confidence score (bino score), between TSC2 and TSC1 and TSC1 and TBC1D7. We found the strongest crosslinks to be highly consistent with the published structural data, indicating that the complex maintained its structural integrity through the experimental process (Fig. 2.8A)[14]. Strikingly, of the 18 crosslinked peptides between Rheb-GDP and TSC2 (Fig. 2.8B), only one crosslink was detected within the GAP domain on TSC2 (K1689) and importantly, had one of the lowest confidence scores. The highest confidence score crosslink (K1748) was detected just outside of the GAP domain on an α-helix that has been proposed to mediate interactions between Rheb-GTP and the TSC complex, likely through TSC2 R1749 and Rheb Q72. Structural modelling has revealed that the two residues are positioned near each other, while biochemical mutational analyses have shown that mutation of R1749 results in reduced GAP activity[14,27]. It is possible that the C-terminal α-helix directly following the GAP domain may be an important structural element that docks TSC2 onto Rheb in the GAP mode and is a maintained interaction during the binding transition from Rheb-GTP to Rheb-GDP.

Figure 2.8. Crosslinks detected between the TSC complex and Rheb-GDP cluster within the dimerization domain and on disordered loop regions of TSC2. (A) Diagram of the top ten highest scoring intermolecular crosslinks within the TSC complex (TSC2-TSC1 and TSC1-TBC1D7). Crosslinker type indicated by color and confidence score (Bino score) indicated by shading. **(B)** Diagram of the 18 intermolecular crosslinks between TSC2 and Rheb. Crosslinker type and confidence score indicated as in (A). **(C)** PDB structure of a single TSC2 monomer within the TSC complex (full PDB TSC complex structure not shown) **(D)** AF2 prediction of human TSC2 monomer in cyan overlaid on the PDB TSC2 monomer. **(E)** AF2 prediction of *S. pombe* TSC2 monomer in purple overlaid on the PDB TSC2 monomer. **(G)** 3D visualization of Rheb-TSC2 crosslinks mapped onto the AF2 human TSC2 monomer with domain shading corresponding to (C).

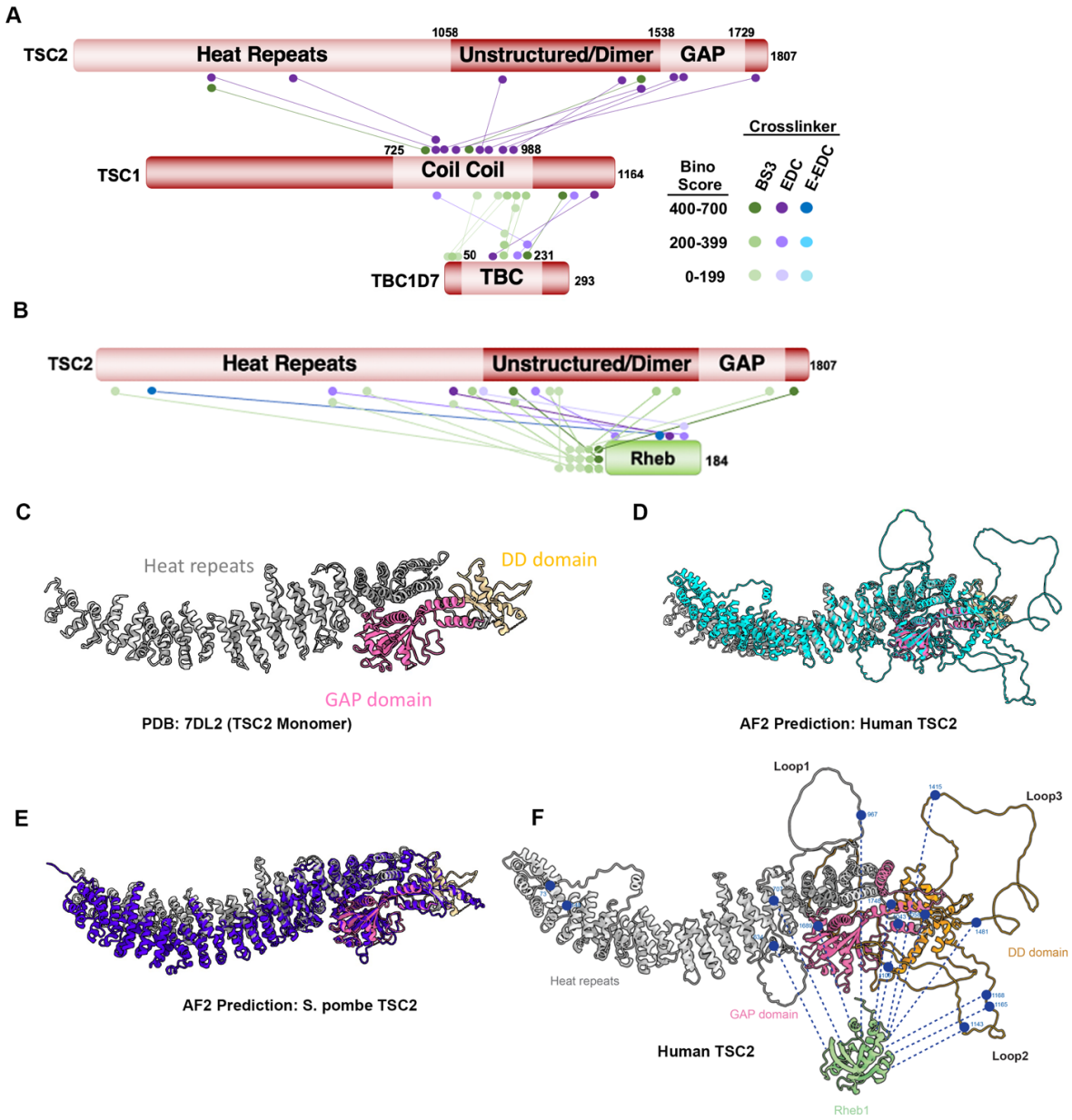


Figure 2.8 (Continued).

Several crosslinks between Rheb-GDP and TSC2 were detected at the N-terminus of TSC2, while many of the crosslinks appeared to cluster within pockets of the HEAT repeats and the unstructured dimer domain (Fig. 2.8B). To visualize the crosslinks in 3D, we mapped the crosslinks onto a TSC2 monomer generated by the protein structure prediction tool, Alphafold (AF2). To validate the predicted structure, we first visually isolated a TSC2 monomer from the TSC Complex Cryo-EM structure (PDB: 7DL2)[14] to visualize TSC2 confirmation in the context of the full complex (Fig. 2.8C). This structure, as predicted, did not resolve many of the regions on TSC2 that contained crosslinked residues to Rheb. To gain access to these unstructured regions, we used the AF2 predicted structure of human TSC2 and overlaid it on top of the 7DL2 TSC2 monomer. The two structures were almost identical outside of the unresolved regions that appeared as floppy loops in the AF2 structure (Fig. 2.8D). We also overlaid an AF2 prediction of *S. pombe* TSC2 onto the 7DL2 TSC2 monomer and observed that the structure was almost identical to the human TSC2 monomer, completely lacking the loop regions that appeared in the AF2 prediction of human TSC2 (Fig 2.8E). Although most of the unstructured/dimer region is missing in *S. pombe*, we observed that the four stranded β-sheet that mediates mammalian TSC2 dimerization[14] is conserved in the S. pombe ortholog, overlaying almost exactly with the human TSC2 β-sheet. Collectively, these structural overlays led us to conjecture that the HEAT repeats, four stranded β-sheet & GAP domain of TSC2 are highly conserved core elements of the TSC complex, while regions that were not resolved appear to have evolved overtime.

Finally, mapping the crosslinks onto the 3D structure of TSC2, we observed that several crosslinks in the dimerization domain were located on the conserved β-sheet and many of the crosslinks clustered on the TSC2 loop regions, which we refer to as Loop 1, Loop 2, and Loop 3 (Fig. 2.8F). In fact, two crosslinks (K967 on Loop 1 and K1106 on Loop 2) were detected twice

with different digest products from the same crosslinked protein. These crosslinks enhanced our confidence in the loop regions as they suggested that the interactions were specific, and not random interactions between Rheb and the flexible and unstructured TSC2 loops. The quantity of crosslinks on the loops, coupled to the dual detection of K967 (Loop 1) and K1106 (Loop 2) promoted us to evaluate the putative role of these TSC2 loops in mediating the interaction between TSC2 and Rheb-GDP.

The unstructured loops on TSC2 are required for the preferential binding of the TSC complex to Rheb-GDP and for suppression of mTORC1 signaling

To probe the potential role of the TSC2 loops in mediating binding to Rheb-GDP, we generated a mutant of human TSC2 (TSC2ΔLoop1/2/3) that looked almost identical to *S. pombe* TSC2. The TSC2 mutant maintained the highly conserved N-terminal HEAT repeats, β−sheet dimerization domain and GAP domain but lacked the three major loops on TSC2. We combined sequence alignments with structural analyses between human and *S. pombe* TSC2 to swap out Loop 1 (917-1020), Loop 2 (1074-1192) and Loop 3 (1226-1496) of human TSC2 with very short sequences from *S. pombe* corresponding to these loop regions (Fig. 2.9A,B). We generated an AF2 predicted structure of the TSC2 human/*S. pombe* chimera and observed structural integrity and proper folding of the mutant using this in silico validation method (Fig. 2.9C).

Despite observing consistently lower expression of TSC2 ΔLoop1/2/3 when co-expressed in cells with TSC1 and TBC1D7, TSC1 and TBC1D7 co-immunopurified with both TSC2 WILD-TYPEand TSC2 ΔLoop1/2/3, indicating that the loop mutant forms an intact TSC complex (Fig. 2.9D). Strikingly, when we fished overexpressed TSC complexes containing either TSC2 WILD-TYPEor TSC2 ΔLoop1/2/3 out of cell lysates with GST-Rheb preloaded

with GDP or GTPγS, we observed that TSC complexes containing TSC2 ΔLoop1/2/3 completely lost their preferential binding to Rheb-GDP. This revealed the importance of the TSC2 loop regions for driving the preferential binding of the TSC complex to Rheb-GDP (Fig. 2.9E).

Significantly, when we reconstituted Hela TSC2 KO cells with either TSC2 WILD-TYPEor TSC2 ΔLoop1/2/3, we observed that despite containing an intact GAP domain, TSC2 ΔLoop1/2/3 was unable to suppress mTORC1 signaling to the same extent as TSC2 WILD-TYPEin this same system. The level of mTORC1 activity in the TSC2 ΔLoop1/2/3 expressing cells was more comparable to the cells completely lacking TSC2, suggesting that the loops on TSC2 are critically important for regulation of mTORC1 signaling through their engagement with Rheb-GDP (Fig. 2.9F).

Figure 2.9. TSC2 loop regions are required for the preferential binding of the TSC complex to Rheb-GDP. (A) Primary structure of human TSC2 with loops 1, 2 and 3 indicated. **(B)** 3D visualization of the crosslinks between Rheb and the TSC2 loops mapped onto the AF2 human TSC2 monomer. **(C)** AF2 predicted structure of the TSC2 ΔLoop1/2/3 mutant. **(D)** TSC complexes from 293F cells expressing either Flag-TSC2 WT, or Flag-TSC2 ΔLoop1/2/3 with co-expression of Myc-TSC1 and HA-TBC1D7 were pulled out of cell lysates by Flag immunoprecipitation and compared to an agarose bead immunopurification control. Quantification of HA-TBC1D7 and Myc-TSC1 binding ratio to Flag-TSC2 indicated in the graph. **(E)** TSC complexes prepared as in (D) were pulled out of cell lysates with GST-Rheb preloaded with GTPγS (500 μm), or GDP (500 μm) and compared to a control pull-down with GST-Kras preloaded with GDP (500 μm). **(F)** Immunoblot of HeLa TSC2 KO cells reconstituted with empty vector, Flag-TSC2 WILD-TYPEor Flag-TSC2 ΔLoop1/2/3 and serum starved for 16 hours prior to cell lysis.

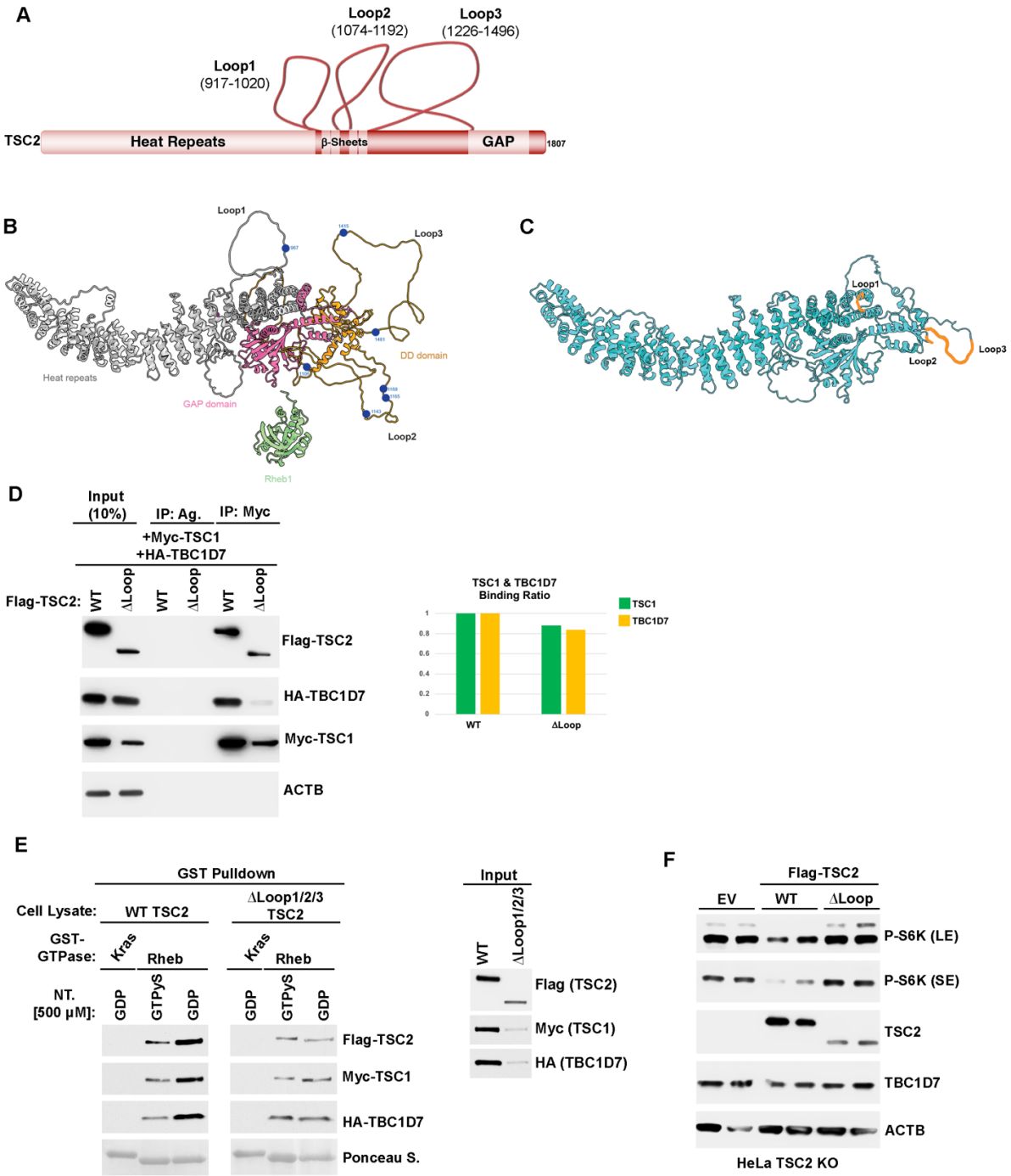


Figure 2.9 (Continued).

Discussion

Here, we describe a novel finding in which the TSC complex preferentially engages Rheb in its inactive, GDP-bound form. Importantly, this interaction occurs outside of the GAP domain, which to date, has been the only described interacting region between Rheb and the TSC complex[4,14-16,23]. Furthermore, we found that the strong interaction between the TSC complex and Rheb-GDP is conserved through *S. pombe*, suggesting that it plays an important role in regulating mTORC1 activation.

Typically, GAP proteins engage GTPases in their GTP-bound state[28], and one prediction from this provocative finding is that the TSC complex may have regulatory functions outside of its canonical GAP domain. Specifically, through its engagement with Rheb-GDP, it is possible that the TSC complex functions as a guanosine dissociate inhibitor (GDI), preventing the exchange of GDP for GTP on Rheb[28]. Interestingly, Rheb has unusually slow GTPase activity, which places it in a highly active GTP-bound state in the absence of the TSC complex[29]. Given this unique biochemical feature of Rheb, it is not surprising that a regulatory function that maintains Rheb in its inactive form is required to prevent hyper-activation of mTORC1 at the lysosome.

Although we were unable to map the interaction between Rheb-GDP and TSC2 to a precise region, our CLMS data prompted us to delete three major loops on TSC2. We determined that these loops are not required for TSC complex assembly but are essential for the preferential interaction between Rheb-GDP and the TSC complex. Significantly, we discovered that these structural elements are required for complete suppression of mTORC1 signaling upon growth factor withdrawal from the cell.

Previous reports have demonstrated that insulin stimulated phosphorylation of TSC2 by Akt inhibits the ability of the TSC complex to regulate Rheb by causing an acute and rapid release of the complex from the lysosome surface, where a fraction of Rheb resides[7]. The loop regions of TSC2 are serine/threonine rich and heavily phosphorylated, with all five of the established Akt sites residing on these loops[20]. While it has been established that spatial localization is a novel mechanism of TSC complex regulation, the molecular intricacies of this mechanism are unknown. We suspect that the TSC2 loops are important structural elements that may mediate lysosomal tethering of the TSC complex, and signal integration through these loops dictate its lysosomal localization and regulation of Rheb nucleotide status. Follow up studies that address TSC complex lysosomal localization in the absence of the loop regions is an essential next step to uncover this potential mechanism.

Importantly, hundreds of missense mutations occurring on TSC2 alone have been identified in patients with the genetic tumor syndrome TSC and the neoplastic lung disorder lymphangioleiomyomatosis (LAM) and have also been found in tumor samples from sporadic cancers[30-32]. Although these mutations are found across the length of TSC2, many of them cluster on the loop regions but are not predicted to be pathogenic. The pathogenic patient mutations that map exclusively to core structural regions on TSC2 impact TSC complex structure and GAP activity, which are essential for its function[33,34]. Meanwhile, we predict that the non-pathogenic mutations that map to the loop regions may disrupt exchange activity, lysosomal localization, or both, however redundancies have been built into these regions such that a single mutation does not impact overall complex function.

Collectively, our findings reveal a novel interaction between the TSC complex and Rheb. We propose a model in which the TSC complex initially engages Rheb in its GTP-bound form

through the GAP domain to stimulate the conversion of Rheb-GTP to Rheb-GDP, and subsequently engages Rheb-GDP through a GAP independent domain thereby maintaining Rheb in an inactive state which we refer to as the "association mode".

Curiously, the dual binding modes between the TSC complex and Rheb is a unique GAP feature shared only, to our knowledge, with a parallel GAP upstream of mTORC1, the GAP for the Rag Heterodimer, GATOR1[35,36]. Further structural analyses will help elucidate the dual binding modes of the TSC complex with Rheb, and the switch from the GAP mode to the association mode. Nonetheless, these studies reveal that outside of its established GAP activity, the TSC complex has important but uncharacterized regulatory functions towards Rheb that are essential for proper regulation of the TSC-Rheb-mTORC1 signaling axis. In the following chapter, I will further discuss the significance of these evolutionarily divergent loops, their putative role in signal integration, and future perspectives on methods to uncover the molecular intricacies of this novel Rheb-TSC complex interaction.

MATERIALS & METHODS

Cell Culture

293E, HeLa and MEF ($TSC2^{+/+}$, $TSC2^{-/-}$, $TSC1^{+/+}$, $TSC1^{-/-}$) 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). The 293F suspension cell line was maintained in FreeStyle™ 293 Expression Medium (Gibco, 12338018) with 1% fetal bovine serum (FBS, Sigma). For transient expression of TSC complex components and mutants, 293E and 293F cell lines were transfected with Polyethylenimine (PEI, Polysciences, 23966) for 48 hours before cell lysis.

S. Pombe Cell Line Generation

Gene cassettes containing the Nmt1 promoter and 3X-Flag (for TSC2) or 3X-HA (for TSC1) and the antibiotic resistance markers (Kanamycin for HA and Nourseothricin for Flag) were generated by PCR amplification off the cassette containing plasmids (PMM21 for Flag and and PMM57 for HA) with long oligos that had 80 bp sequence homology upstream and downstream of the TSC2 or TSC1 start site. *S. Pombe* wild-type cell line was transformed with the cassettes and replica plated on antibiotic plates to select for cells containing the inserted cassette. Once cells were selected, they were maintained at 32 °C on YEA selection plates.

Plasmids

S. Pombe Rhb1 and Ras1 cDNAs were synthesized as a gBlock (www.IDT) and cloned into the pGEX-4T2 expression vector derived from pGEX-4T2-Rheb by PCR In-Fusion (Takara 638910). Primers were designed with the In-Fusion Cloning Primer Design Tool (Takara). All

other cDNA constructs are human sequences. pcDNA3-Flag TSC2ΔGAP was cloned by PCR using In-Fusion, the GAP insert was cloned off the pcDNA3 ΔGAP plasmid gifted from D. Kuemmel into the pcDNA3-Flag TSC2 backbone. pcDNA3-Flag-TSC2ΔC was cloned by PCR In-Fusion off of the pcDNA3-Flag TSC2ΔGAP and into the pcDNA3-Flag TSC2 backbone. TSC2ΔLoop1/2/3 was made by insertion site-directed mutagenesis and primers were designed by NEB base changer (https://nebasechanger.neb.com). The plasmids for the TSC complex purification (pDARMO-CMVT-TSC1-3C-TwinStrep, pDARMO-CMVT-Flag-TEV-TSC2, pDARMO-CMVT-TBC1D7) were gifted from K. Rogala.

Cell Lysis and Immunopurification

For protein extract lysates and Flag immunopurification cells were lysed in ice-cold Triton X-100 lysis buffer (40 mM HEPES, pH 7.4, 120 mM NaCl, 1 mM EDTA, 1% Triton X-100, 5% glycerol, 10 mM sodium pyrophosphate, 10 mM glycerol 2-phosphate, 50 mM NaF, 1mM sodium orthovanadate, 1 μM Microcystin-LR (Enzo Life Sciences, ALX-350-012-C500) and 1:100 protease inhibitor cocktail (Sigma, P8340). 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). For immunoprecipitations, lysates were incubated with Protein-A/G Agarose Beads (ThermoFisher, #20421) or anti-Flag M2 Affinity Gel (Sigma, A2220) overnight at 4°C (unless otherwise indicated). Beads were washed five times with Triton X-100 buffer. Input samples were 10% of lysate. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with primary antibodies overnight. Membranes were incubated with either mouse (CST, #7076) or rabbit (CST, #7074) HRP-linked secondary antibodies for 1 hour and were developed using KwikQuant Pro Imager.

Primary antibodies used were: Myc (CST, 9B11), HA (C29F4), Flag (Sigma, F3165), TBC1D7 (CST, 14949), TSC1 (CST, 6935s), TSC2 (CST, 4308), p-S6K1 T389 (CST, 9234), S6K1 (CST, 2708). GST-tagged GTPases are shown by ponceau S. staining (Sigma, P7170).

Bacterial Rheb Lysis

Rheb expressed in BL21 cells was induced for 6 hours at 30°C when OD reached 0.6-0.8. Cell pellets were resuspended in Rheb Resuspension Buffer (50 mM HEPES, 140 mM NaCl2, 1 mM EDTA, 1 mM DTT and 1:100 protease inhibitor cocktail) followed by treatment with 0.25 mg/mL lysozyme for 15 minutes, 0.2% triton-X for 30 minutes and then 2-5 units of Benzonase Nuclease and 12 mM MgCl2 for 30 minutes. Lysis reaction was spun at max speed (17000 RPM) to clear lysates. Lysates were snap frozen and stored at -80°C for long term storage.

S. Pombe Cell lysis

Cells were grown in 50 mL of YEA media at 32°C to OD 2.2-2.4 and pelleted by centrifugation for 1.5 min, 4400 RPM room temperature, washed in 1X TBS and subsequently re-pelleted. Cell pellets were weighed (.12-.15g) and flash frozen in LN2. Pellets were resuspended in 1.5 volumes of CHAPS lysis buffer (see GST-Rheb affinity purification) with the addition of 1 mM PMSF. 3 scoops of 0.5 mm glass beads were added to each sample (5 scoops/0.25g cells) and bead beaten 4 times (30 sec/6000rpm) with 2 min on ice between cycles. Tubes were punctured with 21G1 needles and lysate was spun into FACS tubes (1.5 min/4400rpm/4°C) for 1 min. Lysates were clarified by centrifugation (12000rpm/4°C) for 10 min.

Size Exclusion Chromatography

For the separation of endogenous proteins from HeLa cells, 10 near-confluent 15-cm dishes were washed with cold PBS and lysed in a total volume of 5 mL 0.3% CHAPS buffer (as in GST-Rheb Affinity Purification). Cleared lysate was loaded into a 5 mL sample loop on the FPLC, injected onto the column (HiLoad 16/600 Superdex 200, Cytiva) and eluted with 0.3% CHAPS lysis buffer. Fractions containing the TSC complex and free TSC2 were collected and pooled. For the separation of S. Pombe TSC complex proteins, 100 mL of yeast culture was pelleted and lysed (as in S. Pombe Cell Lysis) and 1 mL of cleared lysates was loaded into a 1 mL sample loop on the FPLC, injected onto the column (Superose 6 increase 10/300 GL, Cytiva) and eluted and collected as described with the mammalian cells. Before each run the system was equilibrated with three column volumes of lysis buffer.

(See protein purification section for specific columns used in purifications)

Protein Purifications

To purify the TSC complex, TSC1, Flag-TSC2 and TBC1D7 were co-expressed in a 2:2:1 stoichiometry in 1 L of 293F cells for 48 hours. Cells were lysed by resuspension in 40 mL lysis buffer (50 mM HEPES, 100 mM NaCl2, 0.3% CHAPS, 50 mM Glu-Arg, 10% Glycerol plus 1 mM TCEP, 500 uM PMSF, ½ cOmplete protease inhibitor tablet, 1 uL Universal Nuclease) followed by 1 hr. rotation in cold room. Lysates were clarified by centrifugation for 1 hour at 17000 rpm, 4°C and passed through a 100 uM cell strainer. The complex was immunopurified with 500 uL packed anti-Flag M2 Affinity Gel (Sigma, A2220) (1:1 bead slurry in PBS) for at least 2 hours at 4°C. The bead slurry was run over a PD-10 column fitted with a filter to collect

bead bound complex. The beads were washed on the column with 15 mL low-salt buffer (50 mM HEPES, 150 mM NaCl2, 0.3% CHAPS, 50 mM Glu-Arg plus 500 uM TCEP and 500 uM PMSF), 15 mL high-salt buffer (50 mM HEPES, 150 mM NaCl2, 20 mM MgCl2, 0.3% CHAPS, 50 mM Glu-Arg, 5 mM ATP plus 500 uM TCEP and 500 uM PMSF) followed by 15 mL low-salt buffer. Washed beads were eluted twice in elution buffer (50 mM HEPES, 150 mM NaCl2, 0.3% CHAPS, 50 mM Glu-Arg, 10% Glycerol) containing 0.2 mg/ml 3X flag peptide (Sigma, F4799), rotating at 4°C for 30 minutes. The elution's were pooled and concentrated and loaded into a 0.5 mL loop on the FPLC and injected onto the column (Tskgel G4000SWXL, Tosoh) with elution buffer (25 mM HEPES, 300 mM NaCl2, 0.3% CHAPS, 10% Glycerol and 500 uM TCEP). Protein containing fractions were concentrated and snap frozen in LN2.

To purify GST-Rheb/GST-Kras/GST-Rab5A/GST/Rac1: GST-tagged Rheb was expressed in 1 L of E. coli culture, enzymatically lysed in 20 mL of Rheb lysis buffer (see GST-Rheb bacterial lysis) and subsequently passed through a GST-Trap column (GSTrap HP, Cytiva). The GST-Rheb bound to the column was washed 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) and eluted from the column with 30 mL HBS containing 30 mM glutathione. Fractions containing eluted GST-GTPase were spin concentrated, loaded into a 1 mL loop on the FPLC and injected onto the column (Superose 6 increase 10/300 GL, Cytiva) with Rheb elution buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA). Protein containing fractions were concentrated and snap frozen in LN2.

To purify Rheb, purified GST-Rheb was passed through a desalting column (HiTrap Desalting, Cytiva) into PBS and incubated overnight on ice with Thrombin. Cleaved sample was passed back through the GST-Trap, treated with 1 mM PMSF, loaded into a 1 mL loop on the FPLC and injected onto the column (Superdex 75 increase 10/300 GL, Cytiva) with Rheb Elution buffer. Protein containing fractions were concentrated and snap frozen in LN2.

GST-Rheb Affinity Purifications

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). Cell 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-tagged proteins were captured from bacterial lysates with glutathione-agarose beads (Pierce, PI16101), 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 or GTPgS in loading buffer for 15 min at 30°C with gentle agitation. Following loading, samples were placed on ice and nucleotide binding was locked in by adding 10 mM $MgCl_2$ for at least 5 min. 1 ml of lysate (normalized to 2 mg/mL for cells with overexpressed complex components, and 4 mg/mL for cells with endogenous complex components, TSC1-/- MEF cells were normalized to 8 mg/mL to account for reduced TSC2 levels in these cells) was added to loaded GST-GTPase on glutathione-beads (20 μl 1:1bead/buffer slurry). After addition of the lysate, 5 mM EDTA was

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.

Filter Binding Assay

For the equilibrium binding assay, purified GST-Rheb was loaded in loading buffer (50 mM HEPES, 100 mM KoA, 2 mM MgCl2, 2 mM DTT, 0.1% CHAPS) with GTP[α-^{32}P] or GDP[α-^{32}P] (PerkinElmer BLU006H) for up to two hours on ice and subsequently passed through a nitrocellulous membrane (Biorad, 162-0012) by vacuum suction. The membrane was washed with loading buffer, dried and exposed by phosphorimager.

For the competition assay, purified GST-Rheb was loaded in loading buffer with GTP[α-^{32}P] or GDP[α-^{32}P] for up to two hours on ice with increasing concentrations of cold GTP and GDP and subsequently passed through a nitrocellulous membrane by vacuum suction. The membrane was washed with loading buffer, dried and exposed by phosphorimager.

In Vitro Binding Assays

Purified Rheb or GST-Rheb was loaded with 1000-fold molar excess GDP or GTP in solution with loading buffer (50 mM HEPES, 100 mM NaCl, 5 mM EDTA 0.1% CHAPS) for 15 minutes at 30°C with gentle agitation. Nucleotide loading was locked in with the addition of 10 mM MgCl2 for 5 minutes on ice. Loaded Rheb and purified TSC complex were spiked into CHAPS binding buffer containing 500 uM of excess nucleotide (40 mM HEPES, 120 mM NaCl, 1 mM

EDTA, 0.3% CHAPS, 10 mM MgCl2), mixed gently and incubated on ice for 1 hour. Following incubation, 10% of the reaction was saved for the input sample and the remaining reaction was combined with 450 uL of incubation buffer (40 mM HEPES, 120 mM NaCl2, 1 mM EDTA, 0.3% CHAPS, 10 mM MgCl2) and 10 uL of packed anti-Flag M2 Affinity Gel (Sigma, A2220). Samples were immunopurified for 30 minutes at 4°C and washed 5X with incubation buffer. Beads were heated in 20 µl 1X SDS sample buffer at 70°C for 10 min.

For experiments using DSP (ThermoFisher, 22585), 20-fold molar excess DSP to total protein concentration was used. Samples were crosslinked on ice for 30 minutes following incubation and subsequently quenched for 15 minutes with 50 mM Tris-HCL. 50 mM Tris-HCL was added to the incubation buffer during immunopurification.

AlphaFold prediction and Conservation analysis

The structures of human TSC2, Rheb1, S.pombe TSC2 were predicted using AlphaFold v2.0[37](https://github.com/deepmind/alphafold) with the following parameters: db_present = full_dbs, max_template_date = 2022-03-10.

Cross-Linking Mass Spectrometry (in collaboration with S. Gygi Lab)

Purified Rheb (16.2 uM) loaded with GDP (as in the in vitro binding assay) and purified TSC complex (324 nM) were incubated in 80 uL of Binding Buffer (as in in the in vitro binding assay) for one hour on ice. Following incubation, the reaction was split into three tubes and subjected to 1 hour of crosslinking with either Extended EDC (100 mM), EDC (100 mM) and BS3 (2 mM) at room temperature. Following crosslinking, the proteins were digested with

trypsin and the resulting peptides were analyzed by mass spectrometry. See (Ref[38]) for complete details on crosslinking procedure and subsequent analysis.

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Chapter 3:

Discussion and Future Directions

Overview

In this dissertation, I investigated a unique biochemical feature of the TSC complex whereby it preferentially engages the GTPase Rheb in its inactive, GDP-bound form. First, I validated reproducibility and specificity of this interaction, which had been initially reported in a previous study from our lab. I established that the preferential binding for Rheb-GDP was not due to differences in guanylate affinity, and ruled out contributions from additional experimental factors such as the host purification system for Rheb, the nature of specific nucleotide analogs and the presence of epitope tags on exogenously expressed proteins. From these results, I concluded that the preferential binding between the TSC complex and Rheb-GDP is an inherent biochemical feature of the TSC complex. Next, using both overexpression and genetic systems I established that TSC complex binding to Rheb-GDP occurred through TSC2 within the complex and that engagement of TSC2 with TSC1 is required for the interaction. I discovered that this binding interaction is evolutionarily conserved between the *S. pombe* orthologs of the TSC complex and Rheb. I subsequently determined that the interaction is direct, as the preferential binding property can be recapitulated in a reconstituted system using purified TSC complex and Rheb. Using TSC2 mutants that lacking its Rheb-GAP domain, I found that binding between the TSC complex and Rheb-GDP occurs through a GAP-independent domain on TSC2. Given the complexity, size, and interconnectedness of the TSC complex, revealed by a recent partial cryo-EM structure, I chose to use CLMS to broadly map regions on TSC2 that may be interacting with Rheb-GDP. I detected many crosslinks between TSC2 and Rheb on predicted unstructured "loop" regions between the N-terminal HEAT repeats and C-terminal GAP domain of TSC2. To investigate the putative role of these loops in mediating the established binding property, I replaced the three major loops with short corresponding sequences from *S. pombe*, which lack

these loops. I found that when these loops were removed from TSC2, it lost its ability to engage Rheb-GDP, despite forming intact TSC complexes. Importantly, in contrast to wild-type TSC2, the loop mutant was unable to suppress mTORC1 signaling upon growth factor withdrawal, despite having an intact GAP domain. These results revealed that the unstructured loop regions on human TSC2 are important structural elements that are required for the engagement of the TSC complex with Rheb-GDP and provide a second layer of TSC complex regulation of Rheb and mTORC1 to its canonical GAP activity.

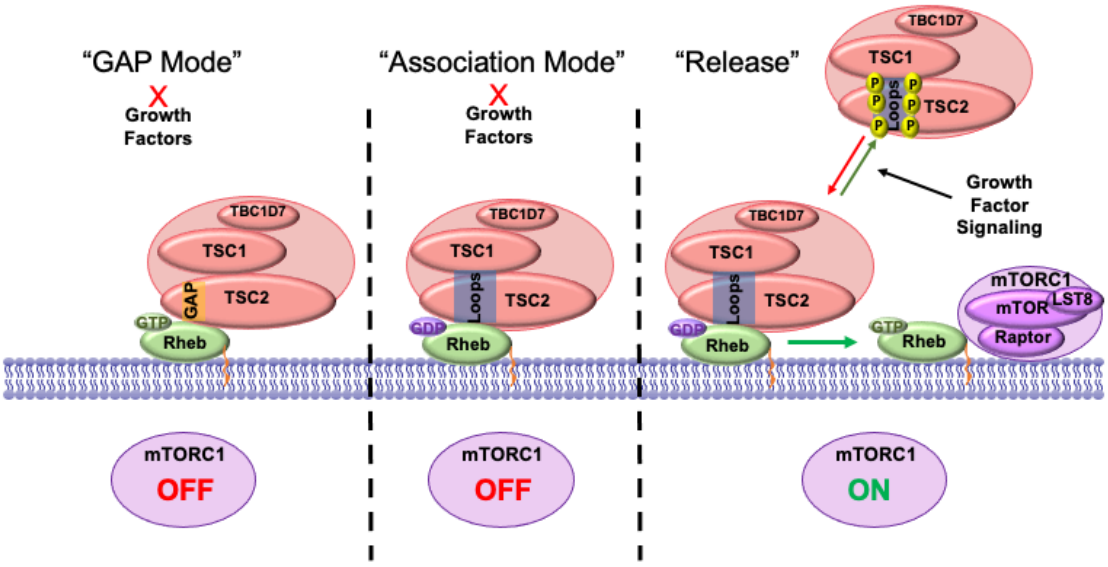


Figure 3.1. Graphical overview of the dissertation.

Future perspectives on the "GAP mode" and "association mode" of TSC2

The TSC complex acts a major node of signal integration, receiving growth signals from the intra- and extracellular environment to control the nucleotide-bound status of Rheb and, ultimately, the activation state of mTORC1. Most signals characterized to date that impinge on the TSC complex modify its ability to regulate Rheb primarily through phosphorylation events on TSC2. Indentified phosphorylation sites on TSC2 span the length of the protein (www.phosphosite.org), and many of the upstream protein kinases that phosphorylate these sites have been identified, including Akt, ERK, RSK, PKC, GSK3, CDK1, AMPK and others[1-8]. Phosphorylation on TSC2 by these kinases can both promote or inhibit the ability of the TSC complex to regulate Rheb and mTORC1. Growth factor signaling through Akt, ERK, RSK, and PKC have all been reported to inhibit the TSC complex and thus activate mTORC1 signaling. Conversely, depletion of intracellular energy or oxygen transmit signals, at least in part, through GSK3 and AMPK to TSC2, which promote the ability of the TSC complex to suppress Rheb-mTORC1 signaling[9].

Despite the identification of these signal specific protein kinases modifying TSC2, the molecular mechanisms by which these phosphorylation events modulate TSC complex behavior remains largely unknown. One of the most well characterized kinases as it relates to TSC complex regulation is Akt, which has been established to phosphorylate TSC2 on five sites (Fig. 3.2). As described in chapter one, our lab has demonstrated that insulin stimulated phosphorylation of TSC2 by Akt on these five sites inhibits the TSC complex by causing an acute and rapid release of the complex from the lysosomal surface, where a fraction of Rheb resides[10]. Unpublished work from the Manning laboratory (not included in this dissertation) has found that growth factor signaling through ERK, RSK, and PKC, protein kinases that can also

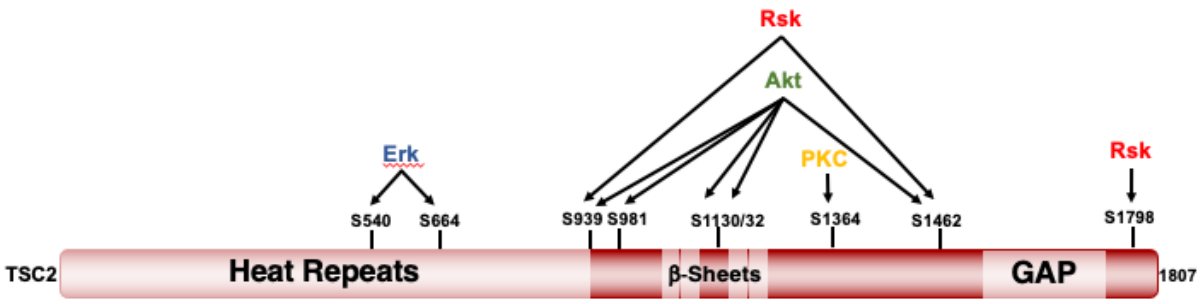


Figure 3.2 Known phosphorylation sites on TSC2. Akt, ERK, RSK & PKC inhibit the ability of the TSC complex to regulate Rheb.

directly phosphorylate TSC2, (Fig. 3.2) also acutely stimulates loss of lysosomal localization of the TSC complex through a mechanism not requiring the 5 Akt phosphorylation sites on TSC2. These data suggest that spatial control of TSC complex lysosomal localization through multi-site phosphorylation is a general mechanism of Rheb-mTORC1 regulation used by diverse growth factor signaling pathways upstream of the TSC complex.

The predicted unstructured loop regions of TSC2 identified in this dissertation to be essential for Rheb-GDP engagement are serine/threonine rich and heavily phosphorylated. Mapping all experimentally identified TSC2 phosphorylation sites from PhosphositePlus (www.phosphosite.org) revealed that most of the sites (70 percent) clustered on the three major loops of TSC2 (yellow), with all five of the established Akt sites residing on these loops as well (green) (Fig. 3.3A). In chapter two, I described the novel finding that these TSC2 loops are required for TSC complex preferential binding to Rheb-GDP and previous work from our lab has established that the TSC complex required membrane localized Rheb to associate with the lysosomal surface[10]. One prediction from these collective findings, is that phosphorylation of residues on these loops influence the ability of the TSC complex to engage Rheb-GDP. Thus, I have investigated whether growth factor signals could influence the interaction between Rheb-GDP and the TSC complex as they do for TSC complex lysosomal localization. Strikingly, I found that when cells were stimulated with either insulin, EGF in the presence of the Akt inhbitor MK2206, or the PKC-activating phorbol ester PMA, the binding of TSC2 in cell lysates with recombinant Rheb-GDP was diminished. Importantly, pre-treatment of the cells with inhibitors of Akt (MK2206) for insulin, MEK (Trametenib) for EGF and PKC (GO6983) for PMA (Fig. 3.3B), restored TSC2-Rheb-GDP interactions to that of the serum starvation condition

Figure 3.3 Phosphorylation on TSC2 reduces the binding interaction between Rheb-GDP and TSC2. (A) All TSC2 phosphorylation sites from Phosphosite residing in the loop regions (yellow) and Akt sites (green) were mapped onto the AF2 predicted human TSC2 monomer (yellow). **(B)** Schematic of the PI3K-Akt and Ras-ERK pathways and their activators and inhibitors. **(C)** HeLa cells were serum-starved for 16 hours prior and stimulated with insulin (100nM) or pretreated with the Akt inhibitor MK2206 (2 uM) for 30 minutes prior to insulin stimulation. Endogenous TSC2 was pulled down from lysates of these cell treatment groups with recombinant purified GST-Rheb pre-loaded with GDP (500 μm), compared to control pull-down with GST-Rab5A preloaded with GDP (500 μm). GST-fused bait proteins were detected with Ponceau S stain. **(D)** Cells were treated and pulled down as in (C) but in the presence of MK2206 (2 uM) with EGF (100 ng/mL) or EGF and Trametinib (5 uM). **(E)** Cells were treated and pulled down as in (C) and (D) but with PMA (100 ng/mL) or PMA and GO6983 (1 uM).

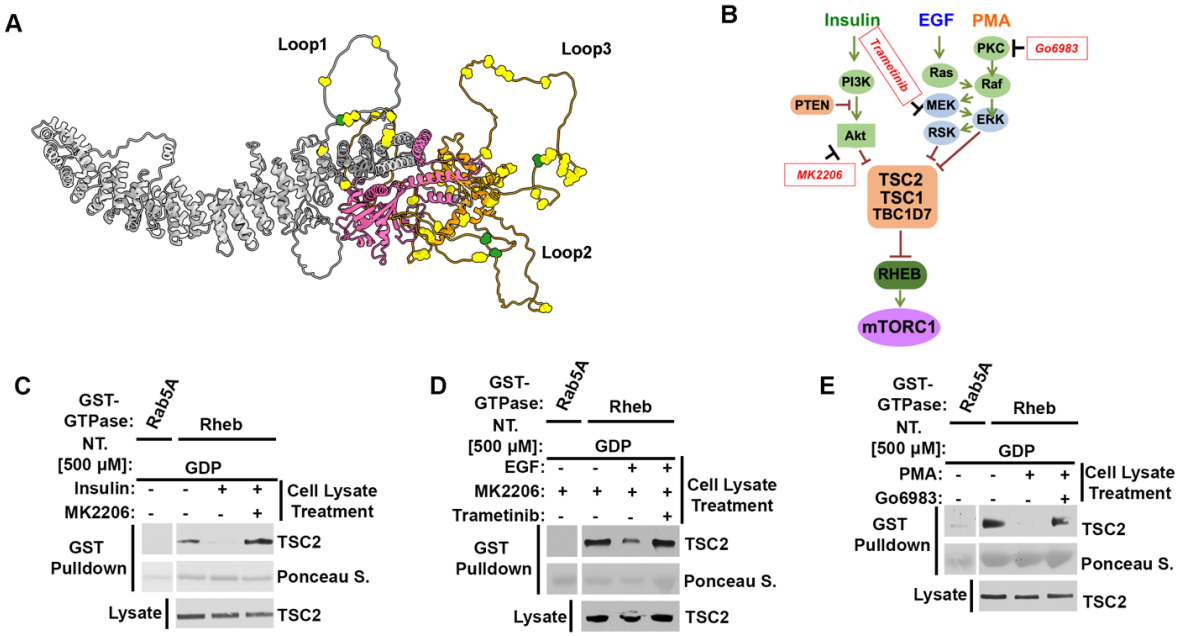


Figure 3.3 (Continued).

(Fig. 3.3C-E). These findings suggest that phosphorylation of TSC2 on distinct sites within these loop regions impacts the interaction between the TSC complex and Rheb-GDP.

The molecular details of TSC complex spatial localization and its regulated release from the lysosome are unknown. My data indicates that the TSC complex strongly engages Rheb-GDP through TSC2 loop regions and that growth factor-mediated phosphorylation events on TSC2 correlate with a decrease in this interaction. These data, coupled to the fact that TSC complex lysosomal localization is also regulated by growth factor signaling, suggests that disruption of the interface between Rheb-GDP and the TSC2 loop regions by growth factor stimulated phosphorylation might be the key event leading to release of the TSC complex from the lysosome—highlighting the critical influence of Rheb binding in spatial control of the TSC complex.

Here, I propose a model in which the TSC complex first localizes to the lysosome in the absence of growth factors and engages GTP-bound Rheb through the GAP domain to stimulate GTP hydrolysis (Figure 3.1, "GAP mode"). Following GAP-mediated conversion to GDP-bound Rheb, the TSC complex engages Rheb through a mechanism requiring the TSC2 loop regions, and potentially other regions conserved in *S. pombe* Tsc2 (Figure 3.1, "association mode"). In the association mode, the TSC complex might have the potential to maintain Rheb in its inactive state, preventing the exchange of GDP for GTP, thereby acting as a GDI towards Rheb. If such GDI activity exist, it would be distinct from established GDIs for small GTPases, as to our knowledge, the TSC complex does not sequester Rheb from its target membranes, as in the case of other reported GDI-GTPase interactions[11]. In fact, in this proposed model, Rheb-GDP reciprocally regulates the TSC complex by tethering it to the lysosome and preventing its release following GAP activity. Site-specific, growth factor-mediated phosphorylation of TSC2 on the

loop regions disrupts the association mode, thereby releasing the TSC complex from the lysosome and allowing Rheb to exchange GDP for GTP and activate mTORC1.

Rheb has unusually slow GTPase activity, which places it in a highly active GTP-bound state in the absence of the TSC complex[12]. This biochemical feature of Rheb makes the GAP activity of the TSC complex critical to Rheb inhibition. I speculate that its sustained engagement with the product of the GAP reaction, Rheb-GDP, perhaps maintains Rheb in its inactive form, preventing the exchange back to GTP-bound Rheb. This speculation is simply a model at this point and needs to be experimentally tested. Studying the intrinsic nucleotide exchange property of Rheb, especially in comparison to other GTPases with known GEFs, will be an important biochemical feature of Rheb to dissect to establish this putative GDI model of TSC complex regulation. However, in support of this model, the TSC2 loop mutant fails to completely suppress mTORC1 signaling in response to growth factor withdrawal from the cell. As TSC2 GAP activity is not predicted to be impacted by replacing the TSC2 loops with the shorter sequences from *S. pombe* Tsc2, I predict that the TSC2 loop mutant fails to engage Rheb-GDP in the association mode at the lysosome, thereby allowing increased local levels of Rheb-GTP and unchecked mTORC1 signaling.

Here, I have proposed a mutual regulation model in which the TSC complex acts as a GDI towards Rheb and Rheb-GDP reciprocally tethers the TSC complex to the lysosome to dictate its spatial localization. However, it is possible that GDI activity of the TSC complex is secondary to the tethering activity of TSC complex to Rheb-GDP, and thus acts as a non-canonical GDI in that it does not sequester Rheb-GDP from the lysosome to prevent GDP exchange. For example, the way in which the TSC complex engages Rheb-GDP in the association mode may prevent exchange by sterically inhibiting GEF activity towards Rheb,

although a Rheb GEF has yet to be reported. Alternatively, in the absence of a GEF, Rheb may constantly exchange GDP for GTP even when engaged in the association mode, but given the proximity of the TSC2 GAP domain, Rheb-GTP might be rapidly turned over to Rheb-GDP. Finally, sustained binding of TSC2 to its product, Rheb-GDP, through a GAP-independent domain, might be important for processivity of the GAP reaction. This would allow a single TSC complex to rapidly convert a local population of Rheb-GTP to Rheb-GDP while staying engaged at the lysosome through the Rheb-GDP tethering activity. Guanine nucleotide exchange assays, in the presence or absence of the TSC complex containing wild-type or loop mutants of TSC2 are needed in the future to properly address these questions. Moreover, Rheb GDP/GTP-exchange assays on liposomes to recapitulate the lysosomal surface could highlight spatial constraints associated with a putative GDI mechanism of the TSC complex and the role of loop phosphorylation in modulating this activity.

Signal convergence and integration through phosphorylation events on the TSC2 loops might enable “fine-tuning” of Rheb’s activation status to match the perceived state of the cell. However, the requirement of the TSC2 loops for TSC complex localization in response to growth factor signaling is correlative and has yet to be directly tested. As such, investigating the lysosomal localization of the TSC2 loop mutant is a critical next step in follow-up to this dissertation work. It is also possible that the loop mutant localizes to the lysosome but to a lesser extent than full-length TSC2. This result would suggest that the loop regions are required for complete localization, but not essential, and other regulators may further mediate spatial regulation of the TSC complex. Furthermore, if the TSC2 loop mutant does localize to the lysosome, it will be important to investigate its responsiveness to PI3K-Akt and Ras-ERK signaling. Finally, it is essential to directly measure the GAP activity of the TSC2 loop mutant to

confirm that this activity is maintained and, thus, not contributing to the inability of this mutant to suppress mTORC1 signaling. In parallel to these studies, a characterization of the binding interaction between the TSC2 loop mutant and Rheb-GDP under these same signaling conditions should also be completed. Finalizing these studies will substantially contribute to our working model of mTORC1 activation by growth factors at the lysosome.

Evolution of the TSC2 loops to coordinate growth factor signaling to mTORC1

The mTOR pathway is highly conserved and serves as a critical regulator of growth in most eukaryotes. In both the budding yeast *S. cerevisiae* and the fission yeast *S. pombe*, two distinct TOR containing complexes have been identified, TOR1 and TOR2. Both complexes contain yeast-specific components, however homologs of Raptor, mLST8, Rictor and mSIN1 have been identified[13-15]. While yeast TORC1 senses and responds to environmental stimuli, the specific environmental cues and upstream pathway components differ for these single cell organisms compared to multicellular organisms[16,17]. Within the nutrient sensing branch GATOR1, GATOR2 and the RAG GTPases (Gtr1/Gtr2) have been identified in both *S. cerevisiae* and *S. pombe*[17,18,19]. However, homologs of the TSC complex (TSC1 and TSC2) have only been identified in *S. pombe*[19,20].

Importantly, while yeast cells possess conserved upstream components involved in mTORC1 regulation, exogenous growth factor signaling evolved with multicellularity, where cells needed to receive information from distinct cell types and tissues. In contrast, nutrient inputs in the form of carbon, nitrogen and phosphate sources appear to be the primary pro-growth inputs into mTORC1 in yeast[19]. Evidence suggests that in yeast, mTORC1 is involved in amino acid sensing, however in *S. cerevisiae* sensing differs from mammals in that amino acids

do not appear to influence the localization of mTORC1 to the vacuolar membrane, the equivalent of the metazoan lysosome[19,21]. Subsequent reports in *S. pombe* found that Gtr1, Gtr2 and mTORC1 also localize to the vacuolar membrane, but like *S. cerevisiae*, amino acids do not dictate the localization status. Amino acids were instead shown to stimulate formation of the Gtr1-Gtr2 heterodimer and promote the interaction between Gtr1 and Mip1 (Raptor)[22].

In general, *S. pombe* is quite divergent from *S. cerevisiae* in that it shares more common features with mammalian cells and is a particular useful model organism for studying conserved cellular processes[23]. Like mammalian Rheb, Rhb1 is essential for growth and under the direct control of TSC complex GAP activity in *S. pombe*[24,25]. Loss of Rhb1 results in growth arrest similar to arrest caused by nitrogen starvation, a phenotype shared by loss of pombe mTOR signaling[26-28]. Moreover, loss of pombe TSC1 and TSC2 results in defects in amino acid uptake and in the nitrogen starvation response, with Rhb1 being genetically epistatic to these effects[24,29,30]. Thus, although the upstream signals are unknown, all evidence indicates that the *pombe* TSC complex acts as a GAP for *pombe* Rheb to control mTORC1 signaling. *S. pombe* TSC2 is highly conserved in both the extended N-terminal HEAT repeats and C-terminal GAP domain but lacks most of the unstructured domain connecting these two regions in mammalian TSC2[9]. I originally hypothesized that if the preferential binding between Rheb-GDP and the TSC complex was conserved, then the GAP-independent binding interface between TSC2 and Rheb could be narrowed down to a highly conserved region between the two proteins. While I discovered that preferential binding for Rheb-GDP is a conserved biochemical property of

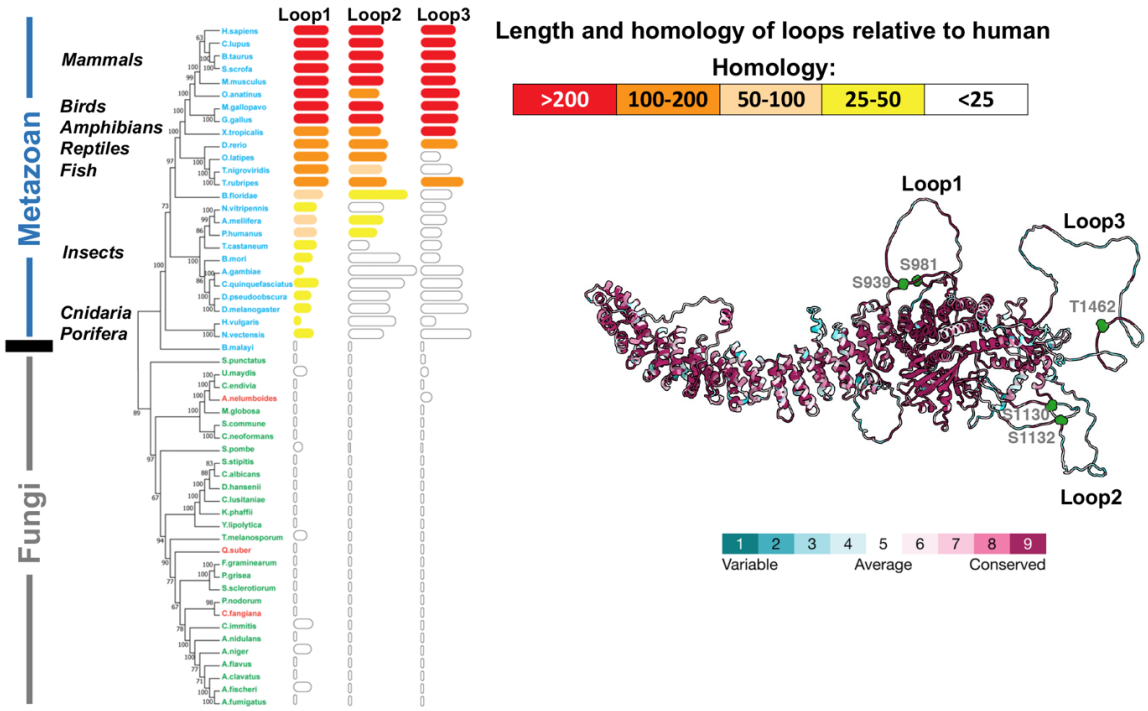


Figure 3.4 Phylogenetic analysis of the TSC2 loop regions. 54 representative species were selected for sequence alignment and phylogenetic tree creation with Clustal Omega. The residue-based conservation analysis and coloring was based on the sequence alignment of the selected 54 sequences and generated using the ConSurf server.

pombe TSC2, the bulk of the binding interactions between human TSC2 and Rheb was found to reside in the divergent loop regions of TSC2 that are almost completely missing in *S. pombe*.

In addition to the loop regions, several crosslinks were detected in the CLMS study on the highly conserved β-sheets found to be extensively involved in TSC2 dimerization in the partial cryo-EM structure of the TSC complex[31]. These β-sheet interactions with Rheb-GDP likely mediate the "association mode" in *S. pombe* but are not sufficient for the interaction in humans, which require the later-evolving loop regions. Interestingly, phylogenetic analysis of TSC2 revealed that all three of the major unstructured loops on TSC2 become increasingly longer and more conserved through evolution from fungi to metazoans (Fig. 3.4). An apparent lower degree of evolutionary constraint on these regions, compared to the core structure of TSC2 (HEAT repeats, dimerization β-Sheets and GAP domain), suggests a role for these loops in metazoan-specific regulation of the complex, rather than core structure and function.

Thousands of mutations of various types have been documented for the *TSC2* and, to a lesser extent, *TSC1* genes in patients with TSC. While many of these variants have obvious functional consequences (e.g., nonsense mutations or deletions), missense mutations affecting amino acid residues spanning the lengths of both proteins, but are more commonly found in TSC2 than in TSC1, often have varying severity of pathogenic consequences[32]. Hundreds of TSC2 missense variants have been publicly reported in the TSC Leiden Open Variation Database (https://databases.lovd.nl/shared/genes/TSC2) and are classified by their probable pathogenicity based on the presence of other TSC mutations in a given patient and functional assays of mTORC1 suppression carried out by the laboratory of Mark Nellist[33-35]. Remarkably, all of the missense variants affecting residues on the TSC2 loop regions are classified as "benign" or "likely benign" with no evidence of functional effects. This is in stark contrast to the numerous

pathogenic variants mapped to the core structure of TSC2, which disrupt the conserved HEAT repeats involved in TSC1 binding or the GAP domain[33]. Mapping the pathogenic variants onto the cryo-EM and alpha-fold structures of TSC2 reveals that the only cluster of pathogenic mutations within the large region between the HEAT and GAP domains affect the highly conserved four-stranded β-sheet that mediates TSC2 dimerization[31]. These pathogenic variants likely disrupt a third core function, TSC2 dimerization, thereby affecting formation of the TSC complex. The lack of observed pathogenicity associated with loop variants highlights the evolutionary flexibility of these regions such that the core form and function of the complex is largely resistant to changes influencing these loops, perhaps with built in redundancies to compensate for mutations.

The *TSC2* gene, located on chromosome 16p15, encodes a transcript of 41 exons. Several functional splice variants of TSC2, expressed in multiple tissues, lack either exon 25 (encoding human 946-988), exon 31 (encoding human 1272-1294) or both[36,37]. These exons are located on loop 1 (917-1020) and loop 3 (1226-1496), respectively, with exon 25 (loop 1) missing from isoform 4 and exon 31 (loop 3) missing from isoform 4 and 5 (https://www.uniprot.org/). Taken together, human genetics support the concept that these loops lack the evolutionary constraints exerted on the core conserved structure of TSC2. I hypothesize that this property has allowed metazoan TSC2 to readily adapt new modes of phospho-regulation to accommodate an increasingly complex and diverse array of growth signals received in animal species.

Future Directions

To date, studies of Rheb regulation by the TSC complex have primarily focused on the TSC2 GAP interaction and mechanism. The work from this dissertation has established that

outside of this canonical interaction, a second Rheb binding site exists within the TSC complex, adding a second layer of regulation to mTORC1. Given the novelty of this interaction, the findings from this work have opened many new questions regarding the dual Rheb binding modes of the TSC complex and their role in controlling mTORC1 signaling. Direct follow up studies, described in more detail previously in this chapter, includes a complete characterization of TSC2 loop contribution to both TSC2 lysosomal localization and Rheb nucleotide exchange. Such studies will address the outstanding questions regarding the regulatory function of the association mode (e.g., spatial regulation and/or GDI activity). Moreover, while I don't predict that the loop regions influence TSC2 GAP activity, measuring this biochemical property will be important for a complete description of the functional effects of the TSC2 loop regions.

It is possible that the TSC2 loops have evolved redundancies such that one loop alone is sufficient to mediate the association mode. While completing my dissertation work, I generated individual TSC2 loop mutants (TSC2ΔLoop1, TSC2ΔLoop2 & TSC2ΔLoop3) and found that deletion of each individual loop does not impact complex formation (data not shown). Moving forward, characterizing Rheb-GDP binding, lysosomal localization, nucleotide exchange and effects on mTORC1 signaling for each of these loop mutants will further refine the association mode domain and its contribution to regulation of the Rheb-mTORC1 signaling axis.

Lacking from this dissertation work is an analysis of Rheb mutants and their ability to engage the TSC complex. Specifically, TSC2 pulldown experiments from cells expressing Rheb constitutively bound to GTP (activating) or GDP (dominant negative) would provide a strong complementary approach to the GST-pulldown assays primarily used in my studies. Identified dominant negative mutants include Rheb D60V, D60K and S20K[38,39], however, these mutants express poorly in cells and have been reported to primarily take on a nucleotide free, rather than

GDP-bound, state—limiting their usefulness. More recently, a crystal structure of Rheb-D60K was determined, and the authors surprisingly found that a GDP was bound to the active site despite no addition of GDP during the protein purification process. However, this mutant was also incapable of binding Mg^{2+}, an effect that should promote less stable binding of guanylates[40]. Thus, a true mutant of Rheb that is in its native state constitutively bound to GDP has yet to be identified for use in studies of TSC complex binding.

The mechanism of TSC2 GAP activity and its interaction with Rheb-GTP have been well characterized. However, it should be recognized that to date, no structure of the TSC complex bound to Rheb, in either nucleotide state, has been resolved. All reported interacting residues between Rheb and the TSC complex are based on structural docking models and mutational analyses alone[31,41]. Given the significant advances in the field of structural biology over the last decade, resolving the structure of the TSC complex bound to Rheb in both nucleotide states is an important and essential path of investigation moving forward. These structures will solidify current models of regulation and significantly expand our understanding of how the TSC complex regulates Rheb through both GAP-dependent and independent mechanisms. Theoretically, well-resolved structures should identify residue level interactions between TSC2 and Rheb in the association mode, proving ample opportunity for mutational analyses to precisely refine this regulatory interface. Finally, these structures will reveal important spatial aspects of the GAP mode and association mode, helping us dissect how the TSC complex makes a switch from engaging GTP-bound to GDP-bound Rheb.

As the preferential binding between Rheb-GDP and the TSC complex is conserved through *S. pombe*, this genetic system can be used to further probe the mechanistic details of the interaction. First, repeating the CLMS study with Rhb1-GDP would be an interesting parallel approach to

identify conserved interacting residues (likely within the β-sheet) outside of the loop regions. Additionally, employing *S. pombe* proteins for cryo-EM analysis of the TSC complex bound to Rhb1-GDP could be an attractive approach for structural studies, given the reduced size (and assumed complexity) of the TSC complex in this genetic setting. Finally, localization studies addressing TSC complex localization to the vacuole would be an interesting avenue of exploration to elucidate the conservation of spatial regulation of the TSC complex in the absence of the divergent TSC2 loops.

Conclusion

In this dissertation research, I investigated a novel biochemical interaction between the TSC complex and Rheb. While GAP proteins typically engage the GTP-bound form of their target GTPase, I found that the TSC complex preferentially engages the GDP-bound form of its target GTPase, Rheb. Mapping this interaction to regulatory loop regions on TSC2, I found that the TSC complex loses its ability to preferentially engage Rheb-GDP in the absence of these loops. Importantly, these loops are not found in the *S. pombe* homolog of TSC2, yet a lack of these loops in mammalian cells prevents full suppression of mTORC1 signaling upon growth factor withdrawal from the cell. This finding suggests that these TSC2 loops have evolved in multicellular organisms to contribute to Rheb and mTORC1 regulation. Collectively, these findings revealed that the TSC complex exhibits two binding modes: the catalytic GAP mode, and regulatory association mode. Strikingly, the dual binding modes of the TSC complex is a unique GAP feature shared only, to our knowledge, with a parallel GAP upstream of mTORC1, GATOR1. The “inhibitory mode” of GATOR1 differs from the association mode of the TSC complex in that it functions to prevent hyperactivation of GATOR1. While the TSC complex

engages GDP-bound Rheb in the association mode, GATOR1 binds GTP-bound RagA/B in the inhibitory mode, thus maintaining the Rags in a prolonged “on” state[42,43]. Nonetheless, the dual binding mode feature of both GATOR1 and the TSC complex suggests a shared mechanism of layered regulation, secondary to GAP activity, that has evolved to acutely regulate mTORC1 signaling in response to fluctuations in growth conditions and signals.

MATERIALS & METHODS

Cell Lines and Chemical Compounds

HeLa 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). For insulin, EGF, and PMA stimulation, subconfluent cells were 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. Inhibitor pretreatments were for 30 minutes with 2 uM MK2206, 5 uM Trametinib or 5 uM GO6983 (Selleckchem, all inhibitors resuspended in DMSO at 1000X stocks)

AlphaFold prediction and Conservation analysis

The structures of human TSC2, Rheb1, S.pombe TSC2 were predicted using AlphaFold v2.0[44](https://github.com/deepmind/alphafold) with the following parameters: db_present = full_dbs, max_template_date = 2022-03-10. For phylogenetic analysis of TSC2, TSC2 sequences from 54 representative species were selected based on the plant and fungi clades of the phylogenetic tree from CLIME[45], and sequence alignment and phylogenetic tree for TSC2 were generated using Clustal Omega. The residue based conservation analysis and coloring were generated using the ConSurf server[46] based on the sequence alignment of the selected 54 sequences.

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