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Recent Progress in the Study of the Rheb family GTPases

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Abstract

In this review we highlight recent progress in the study of Rheb family GTPases. Structural studies using X-ray crystallography and NMR have given us insight into unique features of this GTPase. Combined with mutagenesis studies, these works have expanded our understanding of residues that affect Rheb GTP/GDP bound ratios, effector protein interactions, and stimulation of mTORC1 signaling. Analysis of cancer genome databases has revealed that several human carcinomas contain activating mutations of the protein. Rheb's role in activating mTORC1 signaling at the lysosome in response to stimuli has been further elucidated. Rheb has also been suggested to play roles in other cellular pathways including mitophagy and peroxisomal ROS response. A number of studies in mice have demonstrated the importance of Rheb in development, as well as in a variety of functions including cardiac protection and myelination. We conclude with a discussion of future prospects in the study of Rheb family GTPases.

Keywords

Rheb GTPase; mTORC1; Lysosome; Mouse Study; Structure; Mutants

1. Introduction

Rheb was initially discovered as a GTPase whose expression is induced in rat brain by NMDA-dependent synaptic activity [1]. Since then, it has been found to be highly conserved during evolution and was found to play critical roles in cell growth, cell cycle, autophagy and amino acid uptake [2, 3]. Rheb belongs to the Ras superfamily GTPases [4], monomeric proteins of approximately 21 kDa, that act as molecular switches in a variety of cellular functions. These proteins activate downstream effectors when bound with GTP, but are turned off when bound with GDP. Conserved stretches of amino acids called G-boxes are present in Rheb. Rheb is an activator of mTORC1 that phosphorylates 4E-BP1 and S6K leading to the activation of protein synthesis. These topics were covered in our earlier

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review [3]. A number of important developments have occured since our previous review. First, biochemical and biophysical characterization of Rheb proteins uncovered unique structural features of this protein. Second, cellular localization of Rheb has pointed to its role in lysosomal events. Third, various knockouts of Rheb as well as Rheb knock-in mice have been generated and characterized. These studies provided insight into the biological functions of Rheb. Finally, mTORC1-independent functions of Rheb have also been identified. This review summarizes these new developments.

2. Rheb family proteins

2.1. Structure of Rheb

Rheb proteins, monomeric proteins of approximately 21 kDa, are highly conserved during evolution and Rheb is found from yeast to human. However, no Rheb is found in plants. Lower eukaryotes such as yeast or *Drosophila* have only one gene, but in mammalian cells two different *Rheb* genes have been identified: *RHEB1* and *RHEB2* (also called *RHEBL1*). The gene products share 54% identity and 74% similarity and it is suggested that they perform similar functions. However, their tissue expression profiles differ, with Rheb1 ubiquitously expressed while Rheb2 expression is more limited [5]. In this review, we use Rheb when mentioning Rheb1 except in the section discussing mouse studies (section 4).

Rheb consists of 184 amino acids; the N-terminal 169 amino acids make up the GTPase domain, and the 15 remaining C-terminal residues make up a highly variable region ending in a CAAX motif. The crystal structures of Rheb bound with GTP, GppNHp, or GDP have been determined [6]. Figure 1 shows these structures aligned to highlight the differences between these two structures. The structure of Rheb reveals a high similarity to other small GTPases, with a closer resemblance in structure to Ras and Rap than to Rab5A and RhoA [6]. The switch I region of Rheb undergoes conformational change during GDP/GTP cycle. However, unlike other GTPases, the switch II region maintains a relatively stable structure. The switch II region of Rheb adopts a unique conformation different from the long α-helical conformation seen with other Ras family GTPases. In addition, due to the unique structure of Rheb, Gln64 (corresponding to Gln61 in Ras) is buried in a hydrophobic core and cannot interact with either GTP or the catalytic active site. Another difference with Ras is that a conserved tyrosine residue Tyr35 in Rheb (corresponding to Tyr32 in Ras) shields the phosphate moiety of GTP. These two unique structural features of Rheb suggest that the mechanism of GTPase in Rheb differs from that of Ras. Mazhab-Jafari *et al*. [7] showed that Tyr35 of Rheb maintains Rheb in its highly activated state by inhibiting its intrinsic GTPase activity. A mutation of this residue to alanine (Y35A) results in the increase of intrinsic GTPase approximately 10-fold. Further characterization of the mutant revealed that the GTP hydrolysis involves Asp65 in switch II and Thr38 in switch I. It appears that Tyr35 constrains the conformation of the active site so that the access of Asp65 to the nucleotide binding pocket is inhibited. The Y35A mutant is insensitive to the TSC2 GAP activity.

NMR spectra of Rheb was obtained by analyzing residues 1–169 of Rheb expressed in *E. coli* [8]. ^{1}H-^{15}N heteronuclear single-quantum coherence (HSQC) spectra of GDP- and GppNHp-bound Rheb were compared. Changes in chemical shift were observed with residues in and around the P-loop (residues 10–20) and switch II region (residues 60–65). In

a different study, the structure of Rheb-GDP was determined by NMR [9]. NMR based assay for the analysis of GTPase activity of Rheb was developed [8]. NMR method was also used to characterize Rheb tethered to nanodiscs, monodisperse protein-encapsulated lipid bilayer [10]. NMR measurements showed that the GTPase domain interacts transiently with the surface of the lipid bilayer in two distinct orientations. Membrane conjugation reduced the rate of intrinsic nucleotide exchange without changing GTP hydrolysis.

Ras family GTPases contain a carboxyterminal hypervariable region (HVR) followed by the CAAX motif (A is an aliphatic amino acid, X is a C-terminal amino acid). The presence of palmitoylated cysteines (H-Ras, N-Ras) or a string of basic amino acids (K-Ras) in the HVR, combined with the prenylation of the CAAX motif, target Ras proteins to the plasma membrane [11, 12]. Rheb1 and Rheb2 share a –CAAX motif but lack a polybasic domain or palmitoylated cysteines in the HVR. Rheb1 and Rheb2 become farnesylated at the cysteine, followed by cleavage of the –AAX motif by Rce1, and then carboxylmethylated by Icmt1 [13, 14]. These post translational modifications are required for the association of Rheb to endomembranes [13], [14]. The lack of palmitoylated cysteines or a polybasic domain may explain why Rheb is not associated with the plasma membrane, as the replacement of the HVR of Rheb with the H-Ras HVR led to plasma membrane localization of Rheb [14].

2.2. Rheb mutants

A variety of Rheb mutants have been identified and they are summarized in Table 1. The mutants are grouped into activating mutants, loss-of-function mutants and membrane association mutants. Some activating mutants were based on the identification of *RHEB* mutations in human cancer genome database.

One of the most widely used activating mutants is Q64L that was shown to have increased GTP loading and partial resistance to TSC-GAP [15]. A mutant that exhibits more prominent activation was recently identified by a structure based study [16]. Structural studies of Rheb led to the discovery that the G3-box DxxG motif is involved in the coordination of an Mg^{2+} ion and positions the catalytic H_2O to Cα of glycine and γ-phosphate. This information was used to design substitutions that could affect intrinsic GTP hydrolysis [16]. This study led to the identification of the mutant G63A, which has impaired intrinsic GTPase activity as well as TSC2-GAP mediated GTPase activity. The RhebG63A mutant increased stimulation of mTORC1 signaling compared with wild type Rheb, as revealed by the phosphorylation of S6K.

Mutagenesis studies led to the identification of some activating mutants. Urano *et al.* generated and screened a mutant Rheb library in *S. pombe* to identify hyperactivating mutants of fission yeast Rheb [17]. Introducing these mutations into human Rheb and testing them resulted in the finding that N153S mutant, and S89D mutant to less extent, exhibits increased mTORC1 signaling [18]. This work by Yan *et al*. [18] also identified strongly activating mutants S16N and S16H. Activating mutants were also identified based on the identification of *RHEB* mutations in human cancer genome databases. A large scale genomic analysis of all known cancer genes in 4,742 human cancer samples led to the identification of 33 genes that were not previously known to be mutated in cancer [19]. Among these are genes that are involved in cell proliferation. *RHEB* was identified as one of these genes and

five tumors (two endometrial and three kidney clear cell cancer) were found to carry the Y35N mutation. Expression of Rheb Y35N/C/H mutants resulted in the increase of phosphorylation of S6K [20]. A weak increase of phospho-S6K was observed with the expression of the E139K mutant Rheb.

Mutation of residues in the effector domain (switch I) led to the identification of loss-of-function mutants. Sato *et al.* [21] used an *in vitro* mTORC1 activation assay to test the effect of mutating residues in the effector domain. This led to the finding that D36A, P37A, T38A and N51A mutations decrease the ability of Rheb to activate mTORC1. A total of 65 residues distributed over the entire surface of Rheb were changed to alanine with the aim to find the regions critical for mTORC1 signaling [22]. This study showed that the mutations Y67A/I69A and I76A/D77A result in loss of function. Interestingly, these residues are located in the switch II segment. In contrast, extensive replacement of residues in the switch I region caused relatively little effects on Rheb function. This suggests that some of the switch II residues are involved in downstream effector interaction. Dominant negative mutants of Rheb (S20N, and D60I/K/V) have been identified by mutagenesis studies [23] and inhibition of the mTOR signaling was confirmed [23, 24]. However, these dominant negative mutants tend to have low expression limiting their use in signaling studies.

2.3. Phosphorylation of Rheb

Energy depletion, such as the use of 2-deoxyglucose (2DG), results in the inhibition of mTORC1 as examined by dephosphorylation of S6K1. This event is catalyzed by the cascade of p38β and p38 regulated/activated kinase (p38β-PRAK cascade), as examined by the use of $p38\beta^{-/-}$ and $PRAK^{-/-}$ cells [25]. PRAK phosphorylates Rheb at serine-130 and this phosphorylation is induced by 2-DG and AICAR treatments. Interestingly, the phosphorylation of Rheb resulted in the decreased binding of guanine nucleotides to Rheb. This mechanism is independent of TSC1/TSC2 mediated inhibition of Rheb. Thus, a stress signaling pathway integrates at the level of Rheb protein.

2.4. TSC and RalGAP

The TSC complex consists of TSC1 and TSC2, and TSC2 has a domain with homology to GAP proteins (GTPase activating proteins). In fact, GAP activity against Rheb was detected in a number of studies [15]. The C-terminal end of TSC2 (tuberin) shares significant sequence similarity with Rap1GAP. This RapGAP homologous region is sufficient for the GAP activity against Rheb [26]. Because RapGAPs use an invariant asparagine (Asn-thumb) for its GAP activity instead of an arginine (Arg-finger), TSC2 appears to also use the Asn-thumb mechanism. In support of this idea, arginine-388 that was thought to be responsible for the GAP activity based on mutation in tuberous sclerosis is not required for catalysis but rather affects binding affinity [27]. NMR studies confirmed that TSC provides an Asparagine1643 to stimulate Rheb GTPase activity, and that Arg15 and Gln64 do not play a role in Rheb GTPase activity, but are important for TSC2 complex interaction [8].

TBC1D7 was identified as the third subunit of the TSC1/2 complex [28, 29]. TBC1D7 consists of 293 amino acids (34 kDa). Using an exon-specific deletion strategy, it was shown that TBC1D7 binding involves interaction with TSC1 at exon 22 [30]. TBC1D7

knockdown using siRNA decreased the association of TSC1 and TSC2 resulting in the decrease of Rheb-GAP activity. Mutations in TBC1D7 are associated with Celiac disease, intellectual disability and macrocrania [31]. TBC1D7 is also reported to be a GAP for Rab17 [32]. The TSC1/TSC2 complex is structurally similar to the RalGAP complex that consists of RalGAPα and β [33]. A recent report shows that overexpressed TSC proteins can form a heterodimer with RalGAP and this could explain that the loss of TSC1-TSC2 results in the activation of RalA/B [34], suggesting a crosstalk between the Ral signaling and the mTOR signaling.

2.5. Regulation of Rheb expression and protein level

Rheb is frequently overexpressed in human carcinoma [35]. A meta-analysis of cancer cytogenetic and transcriptional databases led to the finding that the chromosome 7q36.1-q36.3 harboring the *RHEB* gene is frequently amplified in diverse human cancer histologies. Increased *RHEB* expression was observed in liver, lung and bladder cancers. Mircoarray database mining supported a statistically significant association between high-level *RHEB* mRNA upregulation and breast cancer progression. Further support for the significance of Rheb in oncogenic transformation comes from the study that examined the effects of Rheb mutant expression on chicken embryonic fibroblasts [36]. The expression of Rheb Q64L and N153T induced morphological changes, including increased size and vacuolization, and conferred upon the cells the ability for anchorage independent growth.

Rheb expression is elevated under various conditions. In NRK-49F cells, a rat kidney interstitial fibroblast cell line, Rheb mRNA and protein expression was markedly induced by the treatment with TGF-β1 [37]. Study on the host inflammatory responses by microRNA-155 (miR-155) led to the finding that miR-155 regulates Rheb expression [38]. This occurs by the binding of miR-155 to the 3′-untranslated region of *RHEB*. *Mycobacterium tuberculosis* infection leads to enhanced expression of miR-155. This then leads to accelerated autophagic response in macrophages resulting in elimination of mycobacteria. A similar Rheb targeting mechanism is shown to operate in the bacterial clearance in *Pseudomonas aeruginosa* keratitis during corneal infection [39]. Rheb expression is upregulated in immortalized human hepatocytes upon HCV infection [40]. In addition, expression of TSC1 and TSC2 was decreased in infected cells. Hydrogen peroxide promotes ubiquitination and degradation of Rheb in GSH-depleted RAW 264.7 cells resulting in Beclin1-independent autophagic cell death [41].

3. Cellular localization of Rheb

3.1. Lysosomal localization of Rheb

Initial studies using overexpression of Rheb provided hints about their localization on perinuclear and vesicular structures [5, 14, 42]. Co-localization with Rab7 was reported [43]. Later studies showed that this structure represents lysosomes based on the use of a lysosomal marker LAMP2 [44, 45]. Lysosomal localization of endogenous Rheb was established by using the antibody that detects endogenous Rheb [45]. As described before, the membrane localization of Rheb is mediated by its farnesylation and postprenylation

modification events including Rce1 cleavage and Icmt mediated carboxyl methylation [13, 14].

Recent findings clarified the role of Rheb in activating mTORC1 signaling at the lysosomal membrane [reviewed in [46, 47]. A general scheme is summarized in Figure 2. On the lysosomal membrane, two different complexes come together. One type of complex contains mTORC1 which is localized to the lysosome by the action of a heterodimeric GTPase Rag [43], [48]. Rag consists of RagA/RagC or RagB/RagD. The binding of Rag to the lysosomal membrane requires Ragulator that consists of multiple proteins including p18, p14 and MP1. The other complex is TSC/Rheb that is localized by the help of a farnesyl group on Rheb. Co-localization of Rheb and mTORC1 enables activation of mTORC1 by Rheb. Further insight into the regulation of these lysosomal events was provided by the recent reports that addressed signals affecting localization of these proteins [45, 49]. In the study by Menon *et al*. [45], it was shown that insulin causes dissociation of the TSC complex and release of TSC from lysosome, resulting in the activation of Rheb. On the other hand, amino acid starvation did not affect lysosomal localization of TSC2. Since amino acid starvation affects mTORC1 localization, it is suggested that the insulin signals through TSC/Rheb, while amino acids signal through Rag and mTORC1. However, Demetriades *et al.* [49] reported that Rag GTPase binds TSC2 and that this binding is stimulated by amino acid starvation. Further work should reveal the interplay between two different signals that converge on the lysosomal membrane.

3.2. Localization of Rheb to other cellular compartments

In rat liver (FAO) cells, Rheb and TSC1 and TSC2 were shown to localize to the peroxisome, as examined by its localization with the peroxisomal membrane protein 70 (PMP70), and by Western analysis of the peroxisomal fraction of cells [50]. The peroxisomal localization of Rheb, TSC1, and TSC2 is important for ROS induced mTORC1 signaling while peroxisomes are not important for amino acid sensing. Localization of these proteins is dependent on increased ROS, and that this localization inhibits mTORC1 and promotes autophagy.

Rheb has also been shown to localize to the mitochondria, as observed by Rheb colocalization with mitotracker and by Western analysis of mitochondrial fraction of cells [51]. Rheb has also been implicated in regulating mitophagy, which is the autophagosome-mediated degradation of mitochondria (reviewed in [52]). Mitophagy is induced under several conditions, two of which pertain to Rheb: hypoxia and elevated oxidative phosphorylation. Two key proteins upregulated in hypoxic and mitophagic conditions, BNIP3 and BNIP3L, have been shown to bind Rheb [53]. Li *et al.* [53] showed the binding between Rheb and BNIP3 required proper membrane localization of both proteins, because disruption of the transmembrane domain of BNIP3, or the use of an isoprenylation-defective Rheb (C181S), abolished BNIP3-Rheb interaction. Overexpression of BNIP3 decreased Rheb GTP/GDP ratio by 30% and resulted in mTORC1 suppression, as measured by a decrease in S6K and 4EBP1 phosphorylation. This is consistent with the observation that hypoxic conditions suppress mTORC1 activity [54]. In another study, overexpression of Rheb led to an increase in BNIP3L as well as a decrease in mitochondrial mass [55]. This

study showed that increased oxidative phosphorylation caused Rheb to localize to the outer mitochondrial membrane (OMM), where it binds BNIP3L and LC3-II (an autophagosomal membrane marker). The localization of LC3-II to the OMM via Rheb stimulates the autophagosome formation and subsequent mitophagy of the mitochondria.

4. Knockout and knock-in mouse studies of Rheb

Various mouse studies on *Rheb* have been carried out. Table 2 summarizes these studies.

4.1. *Rheb1* is essential for murine development

Mouse knockout studies showed that germline deletion of *Rheb1* results in embryonic death between E10.5 and E11.5. After E11.5, no viable homozygous *Rheb1* mutant embryos were identified [56]. On the other hand, heterozygous *Rheb1* embryos developed normally, and are fertile. Germline deletion of *Rheb2* does not affect murine development, as mutant mice matured without any obvious defects. *Rheb1* knockout mice were also generated by inserting loxP sites in the intronic sequences flanking exon 3 and subsequent expression of Cre recombinase in the targeted ES cells leading to the deletion of exon 3 and germ line inactivation of *Rheb1* [57]. Although mice with homozygous deletion of *Rheb1* were born, their viability declined sharply around midgestation at E12.5. *Rheb1*$^{-/-}$ E10.5 embryos tended to be smaller than controls, but their gross morphology looked normal. On the other hand, E12.5 embryos were smaller and had an apoptotic appearance. Thus, *Rheb1* is essential for murine development beyond E12.

Impairment of TORC1 activity due to *Rheb* deletion was demonstrated by measuring S6 and 4E-BP1 phosphorylation in E11.5 embryonal lysate of *Rheb1*$^{-/-}$ mice [57]. This analysis also showed increased phophorylation of Akt due to the inhibition of the negative feedback loop. Similar results were obtained by the analysis of MEFs cultured from E11.5 *Rheb1*$^{-/-}$ embryo. Further characterization showed that these cells were reduced in surface area and contained higher percentage of G1 phase cells. In agreement with the role of Rheb as a target of TSC1/2, heterozygous deletion of *Rheb1* led to increased life span of *Tsc*1$^{-/-}$ embryo. *Tsc1*$^{-/-}$ embryo die between E10.5 and E13.5. However, *Tsc1*$^{-/-}$*Rheb1*$^{+/-}$ embryos were observed at E15.5. Thus, *Rheb* heterozygosity extends the lifespan of *Tsc1*$^{-/-}$ embryos. The observed embryos, however, were smaller, apoptotic and developmentally retarded.

Rheb-null cell lines derived from mice provided a valuable tool to further study mTORC1 activation. Groenewood *et al.* [58] reported that, while Rheb1 is essential for the rapid increase of mTORC1 activity upon insulin or amino acid stimulation, a significant amount of mTORC1 activity remains in *Rheb1*-deficient MEFs in the continuous presence of serum. This mTORC1 activity remaining in the *Rheb1*-deficient MEFs is blocked by amino acid depletion or upon energy stress. In addition, MEK and RSK inhibitors interfere with the mTORC1 activity. These results suggest that there is a bypass mechanism for the activation of mTORC1 in the absence of Rheb.

4.2. Rheb1 is required for proper development of cardiovascular system

Hematoxylin-eosin (HE) staining of E11.5 embryos of the *Rheb1*$^{-/-}$ mice revealed that heart development was impaired. In some of these embryos, pericardial hemorrhaging and

thinning of the ventricular walls were observed. Areas of apoptotic cells were observed in *Rheb1*$^{-/-}$ embryos as examined by caspase cleavage, DNA fragmentation and TUNEL assay. These results suggest that Rheb1 is required for proper development of the cardiovascular system and that they die due to circulatory failure in the absence of Rheb1.

Cardiac specific *Rheb1*-deficient mice were generated to investigate the role of Rheb in the heart [59]. By postnatal day 8 to 10, these mice died. Observed changes in cardiomyocytes of the cKO mice included hypotrophy, lack of increase of cell surface area of cardiomyocytes, and impairment of sacromere maturation during the neonatal period. In another study, Cao *et al*. [60] showed that conditional knockout of *Rheb1* in cardiomyocytes resulted in malignant arrhythmias, heart failure and premature death at infant stage. Abnormal heart growth and retarded cardiomyocyte size were detected in the cKO mice. In addition, increased cardiomyocyte apoptosis but not autophagy was detected. In another study, cardiac specific *Rheb1* deletion was shown to exert cardioprotection against adverse cardiac remodeling such as hypertension, myocardial infarction (MI), and transverse aortic constriction (TAC) [61].

The observed effects of *Rheb1* deletion on cardiomyocytes appear to be largely explained by the inhibition of the mTORC1 signaling. In fact, mTORC1 signaling in the heart of the cKO mice was impaired after early postnatal period at days 5 or 8, whereas no significant differences in mTORC1 signaling was detected at day 3 [59]. Furthermore, ablation of 4E-BP1 in *Rheb1*$^{-/-}$ mice resulted in the improvement of cardiac hypertrophy, sarcomere maturation, and survival [59]. Proliferation of cardiomyocytes occurs during fetal life, however, they stop proliferation soon after birth. Increases in cardiac mass after birth are achieved by an increase in cell size or hypertrophy. Thus, mTORC1 plays critical role in this process.

4.3. Involvement of Rheb in myelination in postnatal brain development

Rheb1 was deleted in neural progenitor cells by Nes-cre to investigate the role of Rheb1 in brain development [56]. Western blot showed that the level of Rheb1 was reduced by more than 90% in the forebrain of embryos (E15.5, E17.5) and in postnatal brains (P1 and P5). In addition, the level of phospho-S6 (S235/236 and S240/244) in the forebrain was significantly reduced at P1 and P5. While the gross morphology of the brain was preserved at P1 and P5, the weight of brain was reduced by P5-P9. The cortical thickness of the brain was also decreased in the *Rheb1*$^{f/f,}$Nes-cre brain. One of the major changes observed in the *Rheb1*$^{f/f,}$Nes-cre mice was hypomyelination. A decrease in myelin proteins including MBP (myelin basic protein), CNP (2′,3′-cyclic nucleotide 3′-phosphodiesterase), PLP (phospholipoprotein), MAG (myelin-associated glycoprotein), MOG (myelin oligodendrocyte glycoprotein) and MOBP (myelin-associated oligodendrocyte basic protein) was observed in 4-week old brain. The hypomyelination appears to be due to the lack of mature oligodendrocytes. On the other hand, *Rheb1* transgenic mice containing knock-in mutant *RhebS16H*, exhibited enhanced myelination associated with an increase in the number of mature oligodendrocytes.

Effects of Rheb in brain function were studied using AAV transduction of dopamine neurons with constitutively active *RhebS16H*. This study examined neurotrophic effects

using a neurotoxin model [62]. The effects observed include preservation and restoration of nigrostriatal dopaminergic axonal projection.

4.4. Rheb and other cellular functions

Deletion of *Rheb1* in T cells was generated by breeding loxP-flanked *Rheb1* alleles with mice expressing Cre recombinase from the CD4 promoter and enhancer regions [63]. These *Rheb1*-deficient T cells were incapable of generating T_H1 and T_H17 responses *in vitro* and *in vivo*. These cells were less effective in inducing experimental autoimmune encephalomyelitis (EAE) compared with the wild type T-cells. On the other hand, they retained the ability to become T_H2 cells. The above effects of Rheb appear to be due to the failure to activate mTORC1. Deletion of mTORC2 signaling led to the failure to generate T_H2 cells, but their ability to induce T_H1 and T_H17 cells was retained.

Transgenic mice overexpressing *Rheb1* in β-cells have been generated [64]. Activation of mTORC1 signaling in transgenic β-cells was confirmed by immunoblots of phospho-S6 and 4E-BP1. The mice exhibited improved glucose tolerance associated with higher levels of insulin secretion. The mice also showed resistance to hyperglycemia induced by obesity and streptozotocin. Effects of Rheb in spontaneous diabetes were investigated by overexpressing *Rheb1* in β-cells of NOD mice, a type I diabetes model [65]. This study led to the unexpected finding that diabetes progression is accelerated in these mice.

Transgenic mouse lines were generated by targeting murine *Rheb1* expression to basal epidermal keratinocytes [35]. Epidermal Rheb expression was elevated 4-fold and mTORC1 activation was confirmed in transgenic epidermal extracts. Neonatal transgenic mice exhibited phenotypes that include wrinkled black skin, reddened tails and paws, and thickened ears. When histologic analysis was carried out with 3-week-old transgenic back skins, diffuse epidermal hyperplasia was also observed. The mice also presented frequent development of skin tumors associated with stromal angio-inflammatory foci. STAT3 activation was detected in transgenic neoplasia and this was dependent on mTORC1. Furthermore, Rheb1 sensitized transgenic epidermis to squamous carcinoma induction following a single dose of Ras activating carcinogen 7,12-dimethylbenz(a)anthracene.

A mouse kidney fibrosis model was created by unilateral ureteral occlusion (UUO) and the significance of Rheb in kidney fibrosis was examined [37]. Kidneys in these mice developed severe interstitial fibrosis, and activation of mTORC1 was found in the interstitial myofibroblasts from the fibrotic kidneys. Marked enhancement of Rheb and p-4EBP1 staining was detected in tubular cells and in interstitial cells. *Rheb1* transgenic mice in kidney were generated and examined. Increased *Rheb* mRNA and protein levels, as well as activation of mTORC1 signaling, were confirmed in kidney tissue from these mice. Progressive interstitial renal fibrosis was detected in these mice and this effect was inhibited by rapamycin.

Conditional knockout of *Rheb* in mouse germ cells revealed *Rheb* is required for male fertility, but not for female fertility [66]. Analysis of the cKO testes revealed that as mice aged, undifferentiated spermatogonia levels remained constant, while spermatid levels decreased greatly. This led the authors to believe Rheb is required for meiotic progression

during spermatogenesis in an age-dependent manner. The sperm also presented with morphological defects such as amorphous head shape. Female cKO mice did not reveal any defects.

5. mTORC1-dependent and independent functions of Rheb

Many of the above studies reinforce the idea that activation of mTORC1 is a major function of Rheb. A number of phenotypes revealed in the knockout and knock-in mouse studies point to the activation of mTORC1. Rheb has been reported to directly bind mTOR, as overexpressed Rheb could be co-immunoprecipitated with overexpressed mTOR fragment [24]. In *in vitro* studies, purified Rheb can activate mTORC1 in a GTP-dependent manner [21], [67]. Further support for the interaction between Rheb and mTOR comes from FRET-FLIM imaging studies. This method was used to characterize the interaction of Rheb and mTOR [68]. The interaction of these two proteins was revealed from the energy transfer of EGFP-mTOR to DsRed Rheb, suggesting direct physical interaction in living cells.

On the other hand, there are reports of Rheb effects that appear to be independent of mTORC1 activation. Characterization of *TSC* mutant cells uncovered the function of Rheb to inhibit aggresome formation by inhibiting dynein-dependent transportation of misfolded proteins [69], a function independent of mTORC1. Lacher *et al*. [70] reported that Rheb activates AMPK and reduces the level of p27Kip1 in *Tsc2*-null cells and that these effects occur independent of mTORC1 activation. Involvement of Rheb in the regulation of B-Raf kinase activity was suggested from overexpression studies [71], [72]. Saito *et al*., [5] showed that overexpression of Rheb causes the formation of large cytoplasmic vacuole even in the presence of rapamycin. Non-canonical activity of Rheb has been suggested [73].

In addition, various proteins that bind to Rheb have been reported. Binding of Rheb to phospholipase D1 was reported [74]. Rheb binds PLD1 in a GTP-dependent manner and activates PLD1 *in vitro*. Furthermore, overexpression of Rheb in HEK293cells resulted in the activation of PLD1. The authors suggest that PLD1 is an effector of Rheb. Rheb was also reported to interact with BACE1 (β-secretase) in mouse brain, suggesting that Rheb is a new physiological regulator of BACE1 (β-secretase) and amyloid-β generation [75]. BACE1 initiates processing of the amyloid precursor protein to generate amyloid-β, a hallmark event of Alzheimer's disease (AD). It was shown that GTP-bound Rheb interacts with BACE1 resulting in the degradation of this enzyme, mediated by proteasomal and lysosomal pathways. Furthermore, Rheb levels are downregulated in the AD brain. These results point to the significance of Rheb in AD.

PDE4D, the protein responsible for degradation of cAMP, was reported to interact with Rheb [76]. cAMP increases mTORC1 activity through Rheb, as Rheb silencing via sRNA or overexpression of a dominant negative mutant (RhebD60I) diminished cAMP mediated increases in phospho-S6K and pospho-4EBP1. PDE4D binds to Rheb in the same region it binds cAMP, and this interaction is not dependent on the guanine-nucleotide status of Rheb. Increased levels of cAMP disrupt the interaction. The authors propose a mechanism where the increase of cAMP levels causes PDE4D to dissociate from Rheb, thereby allowing Rheb to activate mTORC1 signaling. On the other hand, when cAMP levels are low, excess

PDE4D binds Rheb to decrease mTORC1 signaling. Another incidence where Rheb plays a dual role concerns the Rheb binding protein GAPDH (glyceraldehyde-3-phosphate dehydrogenase) [77]. The nucleotide state of Rheb (GTP verus GDP) did not affect the Rheb-GAPDH interaction. In low glucose conditions Rheb-GAPDH interaction was enhanced and mTORC1 activity was decreased, regulating mTORC1 activity in response to glycolytic flux independently of AMPK and TSC2 [77, 78]. The presence of GAPDH substrate, Gly-3-P, disrupted the interaction between Rheb-GAPDH and increased Rheb-mTOR binding. Atomic force microscopy confirmed increasing amounts of Gly-3-P enhanced GAPDH-Rheb dissociation *in vitro* [79]. These experiments were further validated by another study showing overexpression of the glucose transporter (GLUT1) resulted in a 2.1 fold decrease in Rheb-bound-GAPDH, and a 2.4 fold increase in mTOR associated Rheb [78]. In AMPK inhibitor treated *TSC1*-deficient cells, mTORC1 activity was still inhibited by low glucose. Even a constitutively active Rheb mutant did not rescue mTORC1 activity. Cells lacking functional TSC2 still showed an increase in mTORC1 activity in response to increased glucose uptake. This suggests a model where GAPDH binds and sequesters Rheb away from mTORC1, thus preventing mTORC1 activation under low glucose conditions. As glycolytic flux increases, and thus the intermediate Gly-3-P increases, GAPDH-Rheb interaction is disrupted and Rheb is free to activate mTORC1 signaling.

6. Future Prospects

Rheb is a unique member of the Ras superfamily GTPases. Structural characterization of Rheb revealed a number of important features of this GTPase. TSC1/TSC2/TBC1D7 has been established as a GAP for Rheb and its GAP activity appears to use the Asn-thumb mechanism similar to the RapGAP proteins. Other possible regulators of Rheb such as GDP/GTP exchange factor (GEF) or GDP dissociation inhibitor (GDI) have not been identified and further work is needed to understand how the activity of this GTPase is regulated.

As discussed, a variety of Rheb binding proteins have been identified. It will be important to carry out rigorous characterization of these interactions. Various Rheb mutants may provide valuable reagents for this study. Identification of multiple effectors of Rheb may enrich our understanding of signaling pathways downstream of Rheb.

While many studies provided important insight into various functions of Rheb1, little is known about Rheb2. Mouse knockout studies did not reveal any function of Rheb2, as Rheb2 deletion did not affect organismal viability. In addition, Rheb2 deletion did not exhibit additional effect on mTORC1 and mTORC2 signaling or myelination [56]. Since Rheb2 can activate mTORC1 *in vitro* and in cells, the current thinking is that Rheb2 function is redundant with Rheb1 [21, 23]. Rheb2 function in hematopoietic stem cells (HSC) has been investigated [80], as cDNA libraries showed *RHEB2* to be expressed preferentially in purified HSC populations [81]. Campbell *et al.* [80], showed that overexpression of *Rheb2* enhanced mouse hematopoietic progenitor cell growth. More detailed analysis is needed to understand whether Rheb2 has unique functions.

New Rheb family proteins have been recently identified in *Dictyostelium discoideum* and *Candida albicans. Dictyostelium discoideum* Rheb, DdRheb, shares 52% identity and 100% similarity with human Rheb1 [82]. Cytoplasmic localization to distinct vesicular structures was detected in *D. discoideum* cells, although colocalization studies to determine the exact structures have not been carried out. Rheb is also expressed at all stages of development in *D. discoideum*, including the prestalk and anterior prespore cells. Overexpression of DdRheb results in an increase in cell size. *Candida albicans* Rheb, CaRhb1, shares approximately 41% identity and 65% similarity with *Saccharomyces cerevisiae* Rheb [83]. A sequence of 17 amino acid residues defining the effector domain of Rheb is conserved in CaRhb1. Upon nitrogen starvation, CaRhb1 overexpression reduces filamentation. This appears to be controlled by the decreased expression of ammonium permease through the mTORC pathway. In addition, CaRhb1 is involved in maintaining cell wall integrity. Rheb homologue was identified in three crustacean species of the blackback land crab (*Gecarcinus lateralis*, *Carcinus maenas* and *Homarus americanus*) [84]. Analysis of the expression of this Rheb (GlRheb) showed that GlRheb plays a role in the molt-induced increase in protein synthesis in the claw muscle. This is related to muscle growth in mammalian cells controlled by myostatin. In the future, we can expect more Rheb family members to be identified. Characterization of these new Rheb members should enrich our understanding of the Rheb family GTPases.

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Highlights

- Structural studies of Rheb reveal unique functional features.
- Rheb mutants identified including the ones found in human cancer genome database
- Rheb localization revealed mechanisms of activation of mTORC1 on lysosomal membrane
- Work in mice uncovered diverse roles for Rheb in development

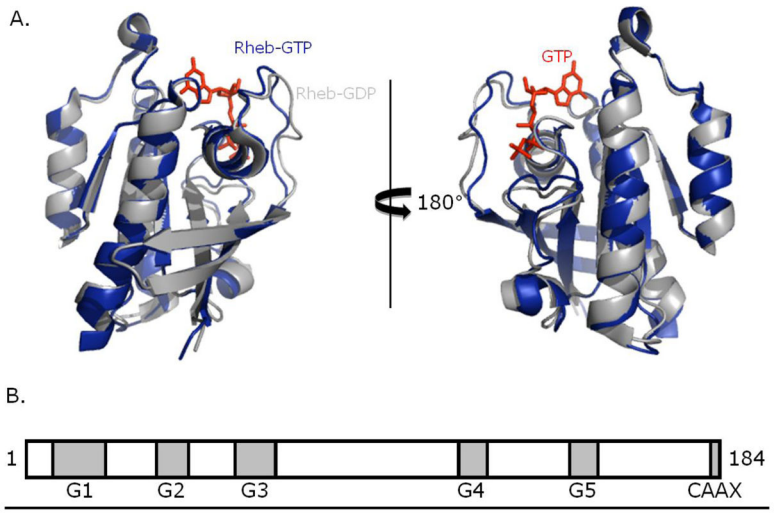

Fig 1. Crystal Structure of Rheb1

A) The crystal structures of Rheb1 bound with GTP (blue) and Rheb1 bound with GDP (grey) were aligned. Only GTP, and not GDP, is shown colored in red. The structures are displayed as rotated 180° around the y-axis. B) Rheb protein, with a length of 184 amino acids, is represented with the approximate locations of the G-boxes 1–5 and CAAX motif. Crystal structures are from the Protein Data Bank (PDB ID: 1XTQ, 1XTS), reference [6].

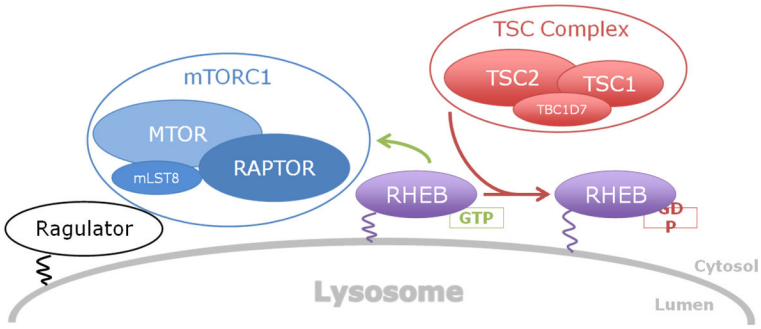

Fig. 2. Rheb stimulates mTORC1 activity on the lysosomal membrane

A schematic representation of Rheb involvement in activation of mTORC1 on the lysosome (protein complexes are not to scale). Rheb localizes to the lysosomal membrane by its farnesylated C-terminal tail. Rheb bound GTP has been shown to stimulate mTORC1 activation at the lysosomal surface, and Rheb bound GDP does not. The TSC Complex, composed of TSC1, TSC2, and TBC1D7, stimulates Rheb GTPase activity and thus inhibits Rheb mediated mTORC1 stimulation.

Table 1

Rheb1 mutations. Listed in this table are *Rheb1* mutations that have been confirmed to exert an effect on Rheb function.

Mutation Type	Amino Acid Position	Mutation(s)	Reference
Activating	16	S16H	[18]
	35	Y35N/C/H	[20]
	63	G63A	[16]
	64	Q64L	[15]
	153	N153T/S	[17, 18]
Activating Mutations Found in Cancer Database	35	Y35N (5x)	[19, 20]
	139	E139K/D/G/* (2X)	[20]
Loss of function	35	Y35A	[7, 17]
	36	D36A	[21]
	37	P37A	[17,21]
	38	T38A	[17, 21, 24]
	39	I39A, K	[17, 24]
	41	N41A	[21, 24]
	65	D65A	[7]
	67, 69	Y67A/I69A	[22]
	76, 77	I76A/D77A	[22]
Dominant Negative	20	S20N	[21, 23]
	60	D60I/K/V	[21, 23]
Loss of Membrane Association	181	C181S	[13, 14]

Table 2

A summary of Rheb mouse studies.

Mice generated to study Rheb function have been listed, along with a brief description of their phenotypes and published references.

	Abbreviation	Tissue	Promoter / Rheb Knockin	Phenotype	Reference
Knockouts	$Rheb1^{-/-}$	All	Actin-Cre	Embryonic apoptotic appearance and death by E12.5	[56, 57]
	$Rheb2^{-/-}$	All	Actin-Cre	Normal Development and Growth	[56]
	$Rheb1^{+/-}$, $Rheb2^{-/-}$	All	Actin-Cre	Normal Development and Growth	[56]
Conditional Knockouts	$Rheb1^{flox/flox}$, Nes-Cre	Neural Progenitor Cells	Nes-Cre	Hypomyleination, decreased brain weight and cortical thickness	[56]
	$Rheb1^{flox/flox}$, αMHC-Cre	Cardiac	αMHC-Cre	Hypotrophy, retarded cardiomyocyte growth, and death by postnatal day 10	[59,60]
	$Rheb1^{flox/+}$, αMHC-Cre	Cardiac	αMHC-Cre	Cardiac protection from TAC and MI	[61]
	T-$Rheb1^{-/-}$	$CD4^+$ T-Cells	Cd4-Cre	Unable to generate TH1 and TH17 cells	[63]
	$Rheb1^{flox/-}$, Vasa-Cre	Germ Cell	Vasa-Cre	Causes male sterility, no effect on female fertility	[66]
Knock-ins	pCAG-Rheb	All	CAG	Activation of interstitial fibroblasts and ECM production, mild fibrotic kidney disease	[37]
	$Rheb1^{k/k}$, Nes-Cre	Neural Progenitor	Nes-Cre / Myc-RhebS16H	Promoted myleination, increased brain weight and cortical thickness	[56]
	$B6^{Rheb}$	Pancreatic β-cells	Rat Insulin Promoter / Flag-Rheb1 WT	Increase in β-cell mass and protection from hyperglycemia	[64]
	NOD^{Rheb}	Pancreatic β-cells	Rat Insulin Promoter / Flag-Rheb1 WT	In NOD background diabetes progression was accelerated	[65]
	K14-Rheb	Keratinocytes	K14 / Myc-Rheb1 WT	Developed multistage epithelial tumorigenesis	[35]