Analysis of Rheb in the cellular slime mold *Dictyostelium discoideum*: Cellular localization, spatial expression and overexpression

PYNSKHEM BOK SWER, POOJA BHADORIYA and SHWETA SARAN*
School of Life Science, Jawaharlal Nehru University, New Delhi 110 067, India

**Corresponding author (Email, saran@mail.jnu.ac.in)*

Dictyostelium discoideum encodes a single Rheb protein showing sequence similarity to human homologues of Rheb. The DdRheb protein shares 52% identity and 100% similarity with the human Rheb1 protein. Fluorescence of Rheb yellow fluorescent protein fusion was detected in the *D. discoideum* cytoplasm. Reverse transcription-polymerase chain reaction and whole-mount *in situ* hybridization analyses showed that *rheb* is expressed at all stages of development and in prestalk cells in the multicellular structures developed. When the expression of *rheb* as a fusion with *lacZ* was driven under its own promoter, the β-galactosidase activity was seen in the prestalk cells. *D. discoideum* overexpressing Rheb shows an increase in the size of the cell. Treatment of the overexpressing Rheb cells with rapamycin confirms its involvement in the TOR signalling pathway.

[Swer PB, Bhadoriya P and Saran S 2014 Analysis of Rheb in the cellular slime mold *Dictyostelium discoideum*: Cellular localization, spatial expression and overexpression. *J. Biosci.* **39** 75–84] **DOI** 10.1007/s12038-013-9405-9

1. Introduction

The Ras homolog enriched in brain gene (*rheb*) is highly conserved in eukaryotes and is present from yeast to mammals (Urano *et al*. 2001). This gene encodes a small GTPase which is closely related to Ras, and exists either in an active GTP-bound state or an inactive GDP-bound state (Clark *et al*. 1997; Im *et al*. 2002). Rheb functions as an antagonist of the TSC (tuberous sclerosis complex) which is an activator of the Rheb-GTPase activity, i.e. a Rheb-GAP (GTPase-activating protein). Cells with a disrupted TSC-Rheb-GAP activity exhibit constitutive activation of the TORC1 signalling. The mechanisms of action of amino acids in the control of TOR signalling and its mechanism in the control of Rheb action are poorly understood. The nutrient-deprived inactivation of TORC1 signalling can be reversed by overexpression of recombinant Rheb. Rheb acts as a positive regulator of TOR signalling by binding to the amino terminal lobe of the mTOR catalytic domain. Constitutive activation of Rheb induces oncogenic transformation via the TOR protein (Jiang and Vogt 2008). Rheb also functions independently of TOR in regulating various aspects of cellular function. We have analysed the Rheb protein in *D. discoideum*. It is a well-characterized eukaryote and a model system to analyse the caspase-independent cell death mechanism. It is a protist growing and dividing mitotically as long as food is abundant. On starvation, these free living amoebae come to common collecting points in response to the chemoattractant cAMP and form multicellular structures which undergo various morphogenetic movements to finally culminate into fruiting bodies consisting of viable spores and dead vacuolated stalk cells. The formation of stalk cells show developmental programmed cell death which is caspase independent. Thus, this organism provides a good model system to study the process of autophagy independent of apoptosis. Rosel *et al*. (2012) have shown that Rheb inhibits phagocytosis in a TORC2-dependent but AKT-independent pathway in this model system. In the present work, we have described the developmental and spatial expression patterns of DdRheb, cellular localization of the gene product and also characterized the Rheb overexpressing strain. Our studies show that DdRheb has all characteristic features of the Rheb family proteins. It is expressed throughout development with higher expression in the prestalk/stalk region and its overexpression leads to an increase in cell size. This is the first detailed study of *rheb* transcript localization during development and could provide new insights into the role of this gene during development.

Keywords. *Dictyostelium*; Rheb; TOR signalling

Published online: 27 January 2014

2. Materials and methods

2.1 *Cell culture and development*

D. discoideum Ax2 cells were grown at 22°C in HL5 medium with constant shaking at 120 rpm. The log phase cells were spread on non-nutrient agar plates at a density of 1×10^7 cells/cm^2 and incubated in humid conditions for multicellular development. Development was synchronized by incubating the plates with the cells at 4°C for 4–5 h and then transferring them to 22°C for further development (Gosain *et al*. 2012).

2.2 *Database search and sequence analysis*

The genomic DNA, cDNA and protein sequences of the putative Rheb were obtained from dictybase online resource (*http://www.dictybase.org*). The protein sequences of known Rheb were obtained from UniProt KB database (*www.uniprot.org*). The domain architectures of the proteins were deduced by Simple Modular Architecture Research Tool, SMART (*http://SMART.embl-heidelberg.de*). Rheb orthologues were searched by Basic Local Alignment Search Tool (BLASTp) at NCBI (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*). Multiple alignments were performed using Cobalt (Constraint-based Multiple Protein Alignment Tool) (*http://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi?CMD=Web*). The same program was used to construct Neighbour joining (NJ) phylogenetic trees with 1000 bootstrap replicates to create a consensus tree.

2.3 *RNA expression of Ddrheb during development*

2.3.1 *In situ hybridization:* Cells were developed on dialysis membrane, supported by KK2 agar, until the desired developmental stages were attained and processed as described earlier by Gosain *et al.* (2012). The spatial expression pattern of *Ddrheb* transcript was studied by *in situ* hybridization with DIG-labelled RNA probe. An approximately 348 bp region of the *rheb* gene from the coding region of the genomic DNA (genomic position 1303 and 1651) was cloned in the vector pBluescript II SK (+) as an XbaI and SacI fragment by using the primers: FP (XbaI) 5′-GACTTCTAGATTTTACAAAAGCAATATTCAATTGGTA-3′ and RP (SacI) 5′-GATCGAGCTCCATTAAAATACAACCTTCTTTTTGTGGAG-3′. The construct thus obtained was confirmed by restriction digestions and sequencing. The construct was digested with SacI to yield antisense templates with respect to the T7 promoter. The digested DNA template was used for *in vitro* transcription of the antisense probe *Ddrheb* RNA.

2.3.2 *RT-PCR:* For semi-quantitative detection of the transcript, RNA was isolated from cells collected at 2 h time intervals from cells developing synchronously on a non-nutrient agar plate using the Trizol reagent (Sigma, USA) as mentioned by Gosain *et al.* (2012). RT-PCR reactions were performed using specific primer sets as given below and *ig7* (*rnl*A) was used as an internal control. The cycle numbers used for amplification were within the linear range of amplification. Primer set for the amplification of *rheb* was FP-5′-GACTGGATCCGCACCACAAAAACATAGAAAGATCTGTGTA-3′ and RP-5′-GACTCTCGAGCATTAAAATACAACCTTCTTTTTGTGGAG-3′. The primer set used for *rnl*A was FP-5′-GGATTCTGCAAAATGGCAAC-3′ and RP-5′-GTCCTCTCGTACTAAAGGAAGG-3′.

2.3.3 *Driving the expression of lacZ under the putative rheb promoter:* A 1021 bp *rheb* intergenic region from Ax2 genomic DNA was PCR amplified using the primers FP (XbaI): 5′-GGCGTCTAGAAAATAAAAACAATTAATGAAAATAAGAAT-3′ and RP (BglII): 5′-GCGAGATCTTCTATGTTTTTGTGGTGCCATTTATA-3′. The reverse primer was designed to include 18 bases downstream of the initiation codon in order to make sure that the right putative promoter region was being amplified during PCR. The product was digested with restriction enzymes XbaI and BglII to facilitate ligation into the vector upstream and in frame with the *lacZ* open reading frame (ORF) sequence of *pEcmAO/lacZ* vector, replacing *ecmAO* promoter to drive the expressions of *lacZ* (stable gal) and generate [*rheb/lacZ*]. It was introduced into Ax2 cells by electroporation and transformants were selected for growth at 100 µg/mL G418 for expression of the reporter gene.

2.4 *Expression of DdRheb under its own putative promoter as a fusion protein with lacZ*

The promoter with ORF region (1.6 kb) of *Ddrheb* was PCR amplified using the following primers FP (XbaI): 5′-GGCGTCTAGAAAATAAAAACAATTAATGAAAATAAGAAT-3′, RP (KpnI): 5′-GACTGGTACCCATTAAAATACAACCTTCTTTTTGTGGAG-3′. The product was digested with restriction enzymes XbaI and KpnI to facilitate ligation into the vector upstream and in frame with the *lacZ* open reading frame (ORF) sequence of *pEcmAO/lacZ* vector, replacing *ecmAO* promoter to drive the expressions of *lacZ* (stable gal) and generate [*rheb/rheb-lacZ*]. It was introduced into Ax2 cells by electroporation and transformants were selected for growth at 100 µg/mL G418 for expression of the reporter gene.

2.5 *β-galactosidase activity in multicellular structures*

β-galactosidase reporter gene expression was visualized in multicellular structures using the modified protocol as described by Gosain *et al.* (2012). After the colour development, the reaction was stopped by washing the cells with Z buffer. The multicellular structures were then photographed under a stereomicroscope (Olympus SZ61).

2.6 *Fusion with reporter protein*

The *Ddrheb* gene was PCR amplified using FP (BamHI): 5′-GACTGGATCCGCACCACAAAAACATAGAAAAGATCTGTGTA-3′and RP (XhoI): 5′-GACTCTCGAGACATTAAAATACAACCTTCTTTTTGTGGAG-3′from the genomic DNA and expressed as a fusion protein with the enhanced yellow fluorescent protein (EYFP) at the C-terminal (Gosain *et al*. 2012). The expression was driven by the constitutive promoter*, actin 15*. The construct was introduced into wild-type Ax2 cells by electroporation and the transformants were selected with 100 µg/ml G418 and was called [*act15*/*rheb-eyfp*]/Ax2 strain Similarly, *Ddrheb* gene was also cloned between the BglII and KpnI sites of the *pD19/lacZ* vector with the amplicon bearing the BamHI and KpnI sites using the primers: FP (BamHI): 5′-GACTGGATCCGCACCACAAAAACATAGAAAGATCTGTGTA-3′ and RP (KpnI): 5′GACTGGTACCCATTAAAATACAACCTTCTTTTTGTGGAG-3′ to create [*D19/rheb-eyfp*]/Ax2 strain. During the cloning process, the BglII site from the vector is permanently lost.

2.7 *Prestalk/prespore isolation*

D. discoideum prestalk/prespore cells were isolated from the migrating slugs by the method of density gradient centrifugation of Ratner and Borth (1983).

2.8 *Immunoblot analysis*

For immunoblotting, samples of 2×10^7 cells were pelleted and boiled in 50 µl SDS sample buffer. 50 µg samples of total protein were size-fractionated on 10% SDS PAGE gels and transferred to nitrocellulose membranes. Membranes were incubated overnight at 4°C with a 1:8,000 dilution of antibody anti-4EBP1 (sc-6025, Santa Cruz Biotechnology Inc) or phospho4EBP1 (Ser65/Thr70) (sc12884, Santa Cruz Biotechnology Inc) or actin (A5060, Sigma) and washed and incubated with 1:10,000 diluted horse radish peroxidase coupled rabbit anti goat or goat-anti-rabbit antibody (Bangalore Genei, India) as required. Detection was performed with the Supersignal chemoluminescence kit (Pierce, USA) according to the manufacturer's instructions.

3. Results

3.1 *In silico analysis of DdRheb*

Homologue of the Rheb protein has been identified in *D. discoideum* which shows all the characteristics of being a GTPase. *Ddrheb* gene is present on chromosome 2. It is a 185-amino-acid-long protein having a molecular mass of approximately 20.75 kDa. The DdRheb protein shares 52% identity and 100% similarity with the human Rheb1 protein. Multiple alignments of deduced amino acid sequences for DdRheb protein with those from other organisms have been shown in figure 1. Like all Rheb proteins, DdRheb also contain G1-G5 boxes, which are short stretches of sequence involved in the recognition of guanine ring and the phosphates of the nucleotide guanine. The key structural features which define the Rheb family are also present in DdRheb. The arginine residue corresponding to the glycine at the 12th position of Ras is conserved in all the Rheb homologues. Very similar effector domain sequences are seen and they all terminate in the CAAX motif (C is cysteine, A is an aliphatic amino acid, and X is the C-terminal amino acid that is usually methionine, alanine, serine, glutamine or cysteine; marked with a green line) that is required for farnesylation. G boxes and lipid modification sites are indicated. Sequence identity was particularly high within the 'G box' motifs (G1–G5) in all the Rheb proteins. The effector domain (switch I region), responsible for interactions with the metazoan target of rapamycin (mTOR), FKBP38 and other proteins (Aspuria and Tamanoi 2004) and C-terminal lipid modification site was also conserved in the DdRheb. Ten out of seventeen amino acids are identical and four are similar in the effector domain. G2 overlaps with the switch I region while the G3 box motif (DXXG) overlaps with switch II region which includes the Walker B motif. Walker A motif is present in the G1 box. The phylogenetic tree shows three distinct clades; where *Dictyostelium* species fall in one, the higher organisms in another and the fungus group in the third clade (figure 2). This kind of distinct clades has also been observed in the phylogenetic tree construction of Ras superfamily members (van Dam *et al.* 2011). Rojas *et al.* (2012) have carried out a detailed analysis of the Ras superfamily and have compared the orthologues of the human Ras superfamily members with 11 proteomes selected on the basis of their relevant moments in eukaryotic evolution, and have shown them also to cluster with their own group members.

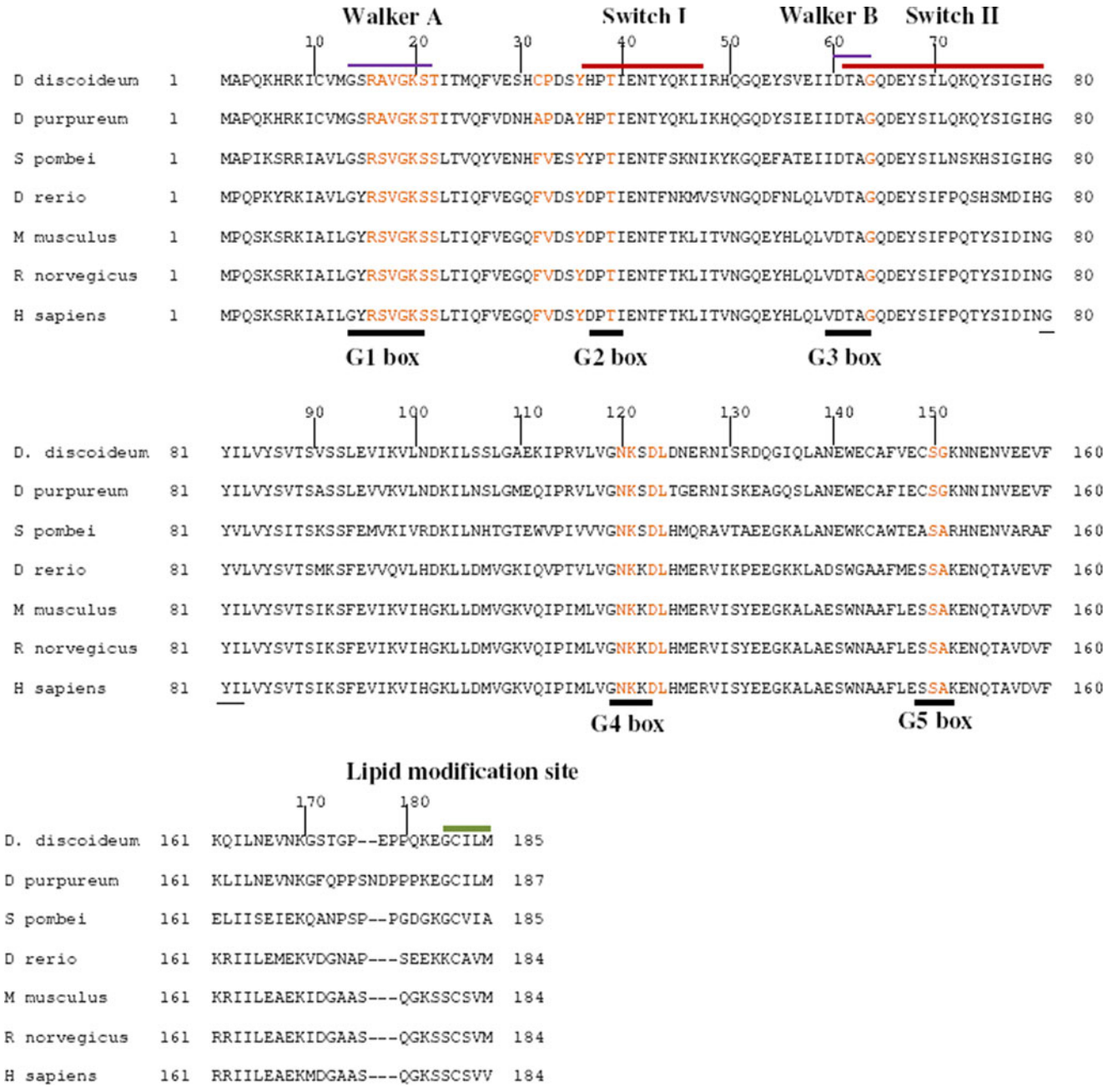

Figure 1. DdRheb is a highly conserved GTPase. Sequence alignment of *D. discoideum* (XP_642878.1), *D. purpureum* (XP_003284036.1), *S. pombe* (NP_595194.1), *D. rerio* (NP_001070216.1), *M. musculus* (NP_444305.2), *R. norvegicus* (NP_037348.1) and *H. sapiens* (BAF82059.1) Rheb proteins by Clustal indicates that DdRheb is a highly conserved Ras-like GTPase. The G boxes (G1–G5) and the CAAX box (green line) are indicated. G boxes and lipid modification site are indicated. G2 overlaps with the switch I region, while the G3 box motif (DXXG) overlaps with switch II region which includes the Walker B motif. Positions of both Walker A and B are also shown.

3.2 *Spatio-temporal expression patterns during development*

Cells in multicellular structures differentiate into two major cell types, the prestalk and prespore cells which are precursors of the terminally differentiated stalk and spores, respectively. We analysed the temporal expression pattern during development by RT PCR. The expression of *ig7* (*rnlA)* was also analysed as an internal control. The results obtained from the analysis demonstrate that *rheb* was

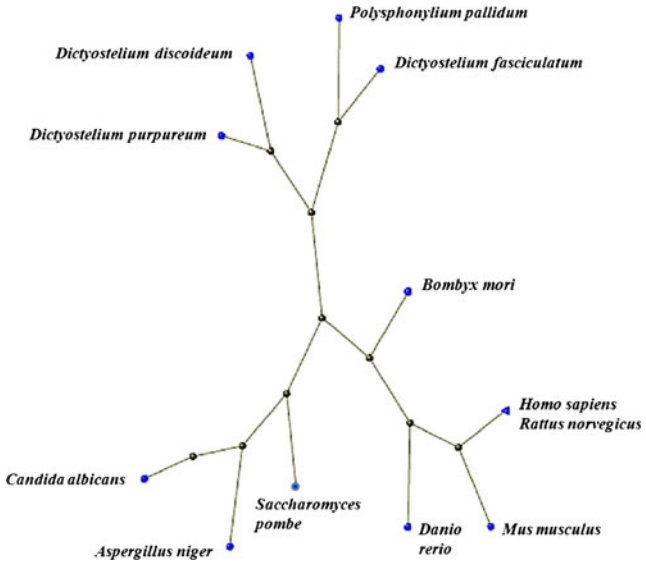

Figure 2. DdRheb shares sequence homology with known Rheb proteins. Neighbour joining (NJ) phylogenetic trees with 1000 bootstrap replicates to create a consensus tree was drawn which shows three distinct clades: where *Dictyostelium* species fall in one, the higher organisms in another and the fungus group in the third clade [*D. discoideum* (XP_642878.1), *D. purpureum* (XP_003284036.1), *D. fasciculatum* (EGG21113.1), *P. pallidum* (EFA80430.1), *S. pombe* (NP_595194.1), *A. niger* (XP_001398466.1), *C. albicans* (XP_723204.1), *B. mori* (NP_001104796.1), *M. musculus* (NP_444305.2), *H. sapiens* (BAF82059.1), *R. norvegicus* (NP_037348.1), *D. rerio* (NP_001070216.1)].

expressed at all stages of development (figure 3A). Densitometry scan shows the transcript level to be slightly higher in the earlier stages with a gradual decrease in later stages of development (data not shown). *In situ* analysis gave similar results showing high expression levels at the aggregate stage. During development, expression was found to be localized largely in the prestalk/stalk region (figure 3B). The prestalk cells in the anterior region are divided into subtypes: pstA, pstAB and pstO (Williams *et al.* 1989). *rheb* is expressed in all prestalk cells and also in the anterior-most prespore cells. In the culminant, pstA cells differentiate into pstAB cells which form the stalk cells (Jermyn and Williams 1991; Gaskell *et al.* 1992). The upper cup (region above the spore head) is mainly composed of both pstAB and pstO cells, while the lower cup is predominantly composed of pstB cells (Early *et al.* 1993; Jermyn *et al.* 1996). The basal disc is mainly composed of pstB cells (Jermyn *et al.* 1996). During culmination we can observe expression in the pstA, pstB and pstO cells. The expression in the stalk tube and basal disc is very distinct. There was very low expression found in the rest of prespore/spore region. Figure 3C (a and b) shows *in situ* hybridization with the sense probe which acts as a control and confirms the results obtained with the antisense probe.

To determine the spatio-temporal pattern of Rheb transcription throughout development, we fused nearly 1000 bp of 5′flanking sequence of *rheb* gene to the *β-galactosidase* reporter gene. The near 1000 bp 5′flanking sequence hosted the putative promoter of the gene as also confirmed by *in situ* hybridization results. Multicellular structures developed from the transformants [*rheb*/lacZ] were stained for β-galactosidase (figure 3D). We found β-galactosidase activity in the prestalk and anterior prespore regions of the slug. This gave near similar results strengthening the fact that the 1000 bp upstream sequence did host the promoter of *rheb*. The spatial expression of the Rheb protein was analysed by driving the expression of *rheb* ORF as a fusion with stable *lacZ* gene under the putative *rheb* promoter [*rheb/rheb-lacZ*]. The expression of Rheb protein (figure 3E) shows high expression in the prestalk/stalk and basal disc regions. Low expression was also seen in the prespore region during development. We observed higher β-galactosidase activity in the above strains developed as compared to that observed with *in situ* hybridization. Although the *rheb* promoter was used for driving the expression in the above cases, this method does not always show the true distribution of the protein because such a big fusion protein (β-galactosidase; 116 kDa) sometimes behaves in a manner different from that of the native protein. We cannot rule out the fact that this may possibly contribute to this effect. In conclusion, we could say that both the Rheb protein and transcript are found largely in the prestalk/stalk cells and also in the anterior-most prespore cells. Low expression is seen in the rest of the prespore/spore regions.

3.3 *Overexpression of Rheb*

We expressed *rheb* as a fusion with the yellow fluorescent protein under the constitutively active promoter *actin15*. Confocal pictures show that Rheb was cytosolic and localized to highly ordered, distinct vesicular structures within the cytoplasm (figure 4). Rheb overexpressing strain [*act15/rheb-eyfp*/Ax2] did not show any obvious alteration in development as compared to the wild type. The fruiting bodies formed were similar to those formed by wild-type cells and there was no difference in the spore viability (data not shown). This indicates that artificial Rheb expression does not have any effect on spore differentiation. This overexpressing strain grows slightly slower than the wild type (data not shown), suggesting that it may have a role to play in cell cycle progression.

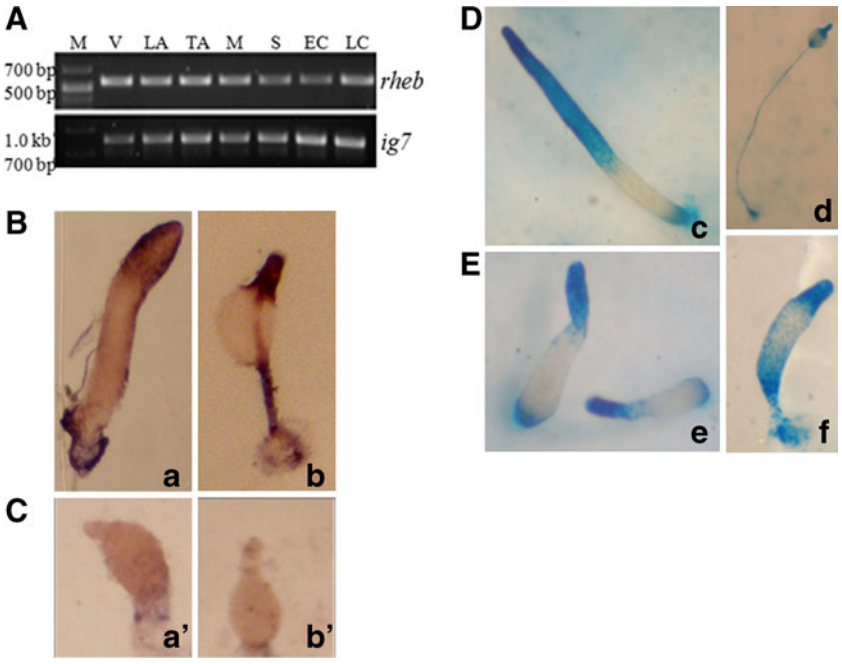

Figure 3. Spatio-temporal expression pattern of Rheb in multicellular structures. (**A**) Temporal expression pattern of *Ddrheb* and its comparison to the control *ig7* during development as studied by RT PCR. Expression is observed at all stages of development. (M, marker; V, vegetative; LA, loose aggregate; TA, tight aggregate; M, mound; S, slug; EC, early culminant; LC, late culminant). (**B**) *In situ* hybridization by *Ddrheb* DIG-labelled RNA probes. The *Ddrheb* antisense probe showed high expression in the anterior prestalk and stalk of slug and culminant (a, b) stages respectively. (**C**) *In situ* hybridization by *Ddrheb* sense DIG-labelled RNA probe as a control (a′, slug; b′, early culminant). (**D**) Spatial expression pattern of Rheb putative promoter as detected by *lacZ* reporter construct [*rheb/lacZ*] in slug (c) and fruiting body (d). The expression was high in the prestalk and stalk regions. (**E**) Spatial expression pattern of Rheb protein as detected by *lacZ* reporter construct in slug (e) and culminant (f). The *D. discoideum* wild-type cells were transformed with vector [*rheb/rheb-lacZ*], where the *rheb* ORF was expressed as a fusion with the *β-galactosidase* reporter gene and driven under the control of its own promoter. The expression is high in the prestalk/stalk regions.

3.4 *Expression of Rheb in the prespore cells*

We next wanted to check the effect of Rheb expression in prespore cells where they are naturally not expressed during development. The growth promoting activity of Rheb is well documented in *Drosophila*, where wild-type Rheb enhances cell replication and induces an increase of cell size and where reduced or deleted Rheb function leads to cell cycle arrest (Patel *et al*. 2003; Saucedo *et al*. 2003; Stocker *et al*. 2003). This probably stimulates the phosphorylation of p70S6 kinase (S6K) and eukaryotic initiation factor 4E(eIF4E)-binding protein (4EBP1), showing it to be regulating the TSC1/2 and mTOR pathway, which still needs to be defined in case of *D. discoideum*. When the expression of Rheb was driven under the prespore-specific D19 promoter, we observed exclusive expression in the prespore region (figure 5A). There were no obvious alterations noticed in development. We then isolated the prespore cells from the migrating slugs and carried out a forward scan using flow cytometry. We compared the sizes of the isolated prespore cells collected from the migrating slugs developed from either Ax2, [*rheb/rheb-lacZ*/Ax2], [*act15/rheb-eyfp/*Ax2] and [*D19/rheb-lacZ*/Ax2] cells. We found the size of the prespore cells isolated from Ax2 and [*rheb/rheb-lacZ*/Ax2] to be comparatively smaller, while the prespore cells isolated from [*act15/rheb-eyfp*] and [*D19/rheb-lacZ*/Ax2] were larger. In the first two cases the Rheb expression is generally not seen in the prespore cells, while in the latter two they are found in the prespore cells. Rheb expression certainly increases the size of the cells. We have yet to confirm if there is any effect on cell cycle progression.

3.5 *Rheb is a component of TOR signalling chain*

Rheb is a component of the phosphatidylinositol 3-kinase (PI3K) target of rapamycin (TOR) signalling pathway and is its positive regulator. The transforming activity of Rheb is

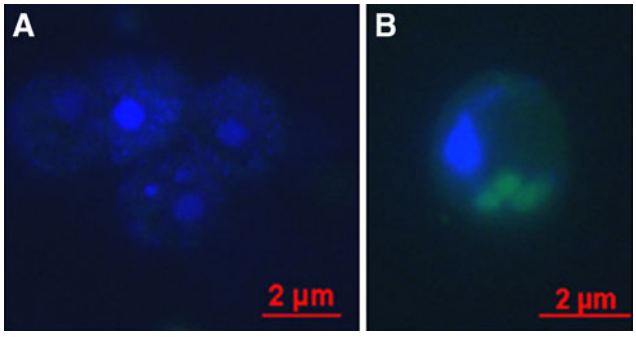

Figure 4. Subcellular localization of DdRheb as studied by fusion with Eyfp. Subcellular localization studies in constitutively expressing [*act15/rheb-eyfp*]/Ax2 cells. (**A**) Blue stain is due to DAPI, a marker for nucleus. (**B**) The fusion protein (green) is localized in the cytoplasm as discrete structures.

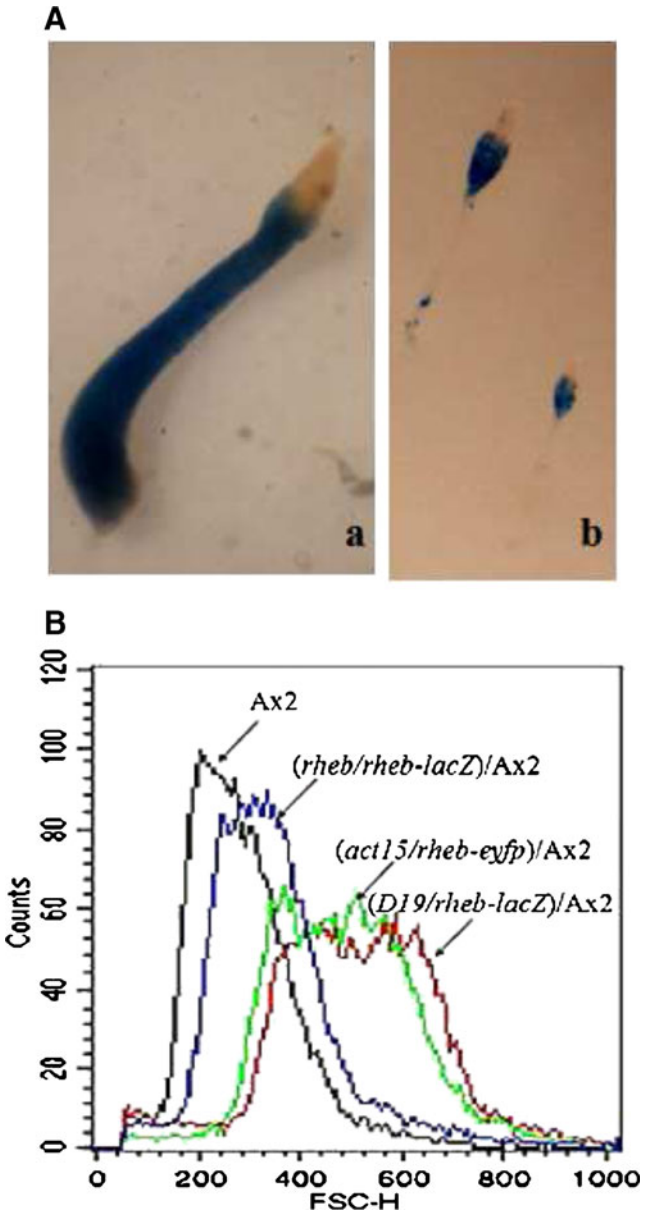

Figure 5. DdRheb overexpression causes increased cell size. (**A**) The DdRheb was ectopically expressed in the prespore region [D19-rheb-lacZ/Ax2]. The slug (a) and the fruiting body (b) shows the β-galactosidase activity (blue colour). (**B**) Forward scan of the isolated prespore cells from the migrating slugs developed from Ax2 [*act15/rheb-eyfp*/Ax2], [*rheb/rheb-eyfp*/Ax2], and [D*19/rheb-lacZ*/Ax2] was run on flow cytometry to analyse their sizes. Overexpression of Rheb causes an increase in the size of the prespore cells.

highly sensitive to the TOR inhibitor rapamycin and is therefore TOR dependent (Hay and Sonenberg 2004). Accordingly, Rheb-induced transformation is correlated with enhanced phosphorylation of the TOR targets S6K and 4EBP1. It is quite well known that there are many more activities of Rheb which are not mediated by TOR. In order to find its role in TOR signalling, we analysed the transcript level of *tsc* and AMP-activated protein kinase, (*ampk*) and the protein level 4EBP1 in these strains both in presence and absence of the drug rapamycin. Treatment with rapamycin inhibits the TOR activity to induce autophagy and regulate protein translation which can be observed by measuring the phosphorylated versus non-phosphorylated 4EBP1 levels. Using compounds designed to inhibit mTOR is a common strategy in the investigation of possible treatments for TSC. Wild-type cells when treated with rapamycin do show a decrease in *tsc* levels but not as significant as that observed with the Rheb overexpressing cells (figure 6A, first panel). TSC2 negatively regulates the activity of Rheb and thereby inhibits mTORC1 signalling. TSC2 subunit is a GTPase-activating protein (GAP) for the Ras-related GTPase Rheb (Inoki *et al*. 2003). When bound to GTP, active Rheb collaborates with TOR to mediate TOR signalling, which includes the phosphorylation of S6K and 4EBP1, two key regulators of protein synthesis. Figure 6A (second panel) shows the *ampk* levels in the same strains under similar conditions. Autophagy is promoted by AMPK, the key energy sensor which regulates cellular metabolism to maintain energy homeostasis. AMPK is sensitive to the cytosolic AMP-to-ATP ratio and responds to metabolic stresses affecting this ratio, such as glucose deprivation, hypoxia, ischemia, heat shock, or oxidative stress. Not surprisingly, AMPK is involved in autophagy induction under these conditions. However, the molecular mechanism underlying how AMPK regulates autophagy is largely unknown although it is generally assumed that AMPK stimulates autophagy by inhibiting mTORC1 at the level of TSC2 (Kim *et al.* 2011) and Raptor (Inoki *et al.* 2012). Under glucose starvation,

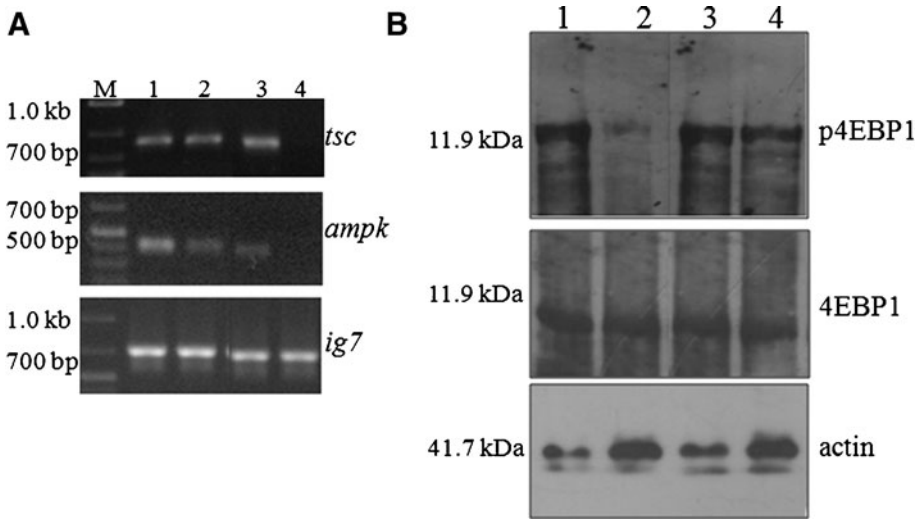

Figure 6. Involvement of DdRheb in the induction of autophagy. (**A**) The transcript levels of *tsc* (panel 1), *ampk* (panel 2) and *ig7* (panel 3, internal control) were analysed in different strains. The wild-type Ax2 strain (lane 1) and the constitutively expressed strain DdRheb (lane 3) were treated with 50 nM rapamyicn for 48 h (lane 2 for wild-type and 4 for Rheb overexpresser) and the level of *tsc2* and *ampk* transcripts were measured. A decrease in the *tsc2* and *ampk* transcript levels were observed in the rapamycin-treated Ax2 strain as compared to the untreated Ax2. A reduction in the transcript levels of *tsc2* and *ampk* was observed in the overexpressed strain of DdRheb, which was more pronounced after rapamycin treatment. (**B**) TOR kinase activity was measured by the phosphorylation of the downstream target 4EBP1. As a positive control Ax2 strain treated with 50 nM rapamycin for 48 h was used to compare against the DdRheb *[(act15/rheb-eyfp)/*Ax2*]* treated with the same rapamycin concentration. The non-phosphorylated 4EBP1 was used as an internal control and the constitutively expressed protein actin as the loading control. A decrease in phospho-4EBP1 was observed in both the wild type and the Rheb overexpressers after rapamycin treatments. [lane1, Ax2 cells; lane2, Ax2 cells treated with 50 nM rapamyicn for 48 h; lane3, (*act15/rheb-eyfp*/Ax2) cells; lane4, constitutively expressed strain DdRheb treated with 50 nM rapamyicn for 48 h].

AMPK promotes autophagy by directly activating Ulk1 through phosphorylation. We observed a decrease after rapamycin treatment in the *ampk* levels. Rheb increases TOR activity and AMPK inhibits TOR activity; therefore, we find the *ampk* levels to be comparatively lower than the wild type, which becomes negligible upon treatment with rapamycin. In Figure 6B, we observe that the phosphorylated 4EBP1 levels decrease upon rapamycin treatment (lane 2) in comparison to the untreated sample (lane 1) in wild-type Ax2 cells. We find similar results with the [*act15/rheb-eyfp*/Ax2] cells (lane 3 and 4). The decreases in the levels are more prominent with the wild-type cells. This suggests that the rapamycin treatment given here does induce autophagy in our studies.

4. Discussion

In the present study we have analysed the Rheb protein during development of *D. discoideum*. Rheb is a known component of the TOR signalling pathway but a direct evidence of their interaction is still lacking. It is proposed that Rheb may bind to and activate TOR-interacting proteins like Rictor, Raptor or LST8 rather than interacting directly with TOR. We have analysed Rheb to determine its role in cell growth and development. The Rheb protein of *D. discoideum* has high identity with orthologs from other species. All Rheb proteins contain G1–G5 boxes, short stretches of sequences involved in the recognition of guanine ring and phosphates of guanine nucleotides (Aspuria and Tamanoi 2004; Tamanoi 2011). Like all Rheb proteins, DdRheb also show the unique characteristics as described by Aspuria and Tamanoi (2004). The similarity of DdRheb to other Rhebs from different organisms suggests that they too may have similar functions. Rheb was expressed at all the stages of development, which is consistent with its ubiquitous expression in other organisms (Aspuria and Tamanoi 2004). Expression of *rheb* was negligible in prespore cells in the developmental phase, indicating that Rheb may not be very essential for these cells. Results obtained from *in situ* analysis suggest that Rheb plays an important role in formation of the stalk, upper cup and lower cup in the developmental phase. It is possible that Rheb is involved in the process of TOR signalling responsible for the process of autophagy and therefore is largely present exclusively in the dying cell

population. Further analysis to determine the target proteins of Rheb in growing cells and developing prestalk cells is in progress. In our studies, we observed the Rheb fusion protein to be localized in distinct structures in the cytosol. It has been earlier shown by Buerger *et al*. (2006) that after a brief association with endoplasmic reticulum, EGFP-Rheb localizes to highly ordered distinct structures within the cytoplasm which show the characteristics of Golgi membranes. Rheb was also reported to be localized with mitochondria (Wang and Proud 2009), along with FKBP38 and mTOR (Ma *et al*. 2008). It has also been shown that GFP-tagged Rheb colocalizes with Rab7, a marker for endosomal and lysosomal structures (Sancak *et al*. 2008). Yadav *et al.* (2013) have shown that mTOR directly interact with Rheb-GTPase and Raptor proteins using fluorescence lifetime imaging. We still need to carry out colocalization studies for a conclusive Rheb localization in *D. discoideum*. The function of Rheb has been extensively characterized *in vitro* but their function *in vivo* is still not well understood. Rheb plays an important role in cell growth and cell cycle progression as was observed in *Drosophila* and yeast (Patel *et al*. 2003; Aspuria and Tamanoi 2004; Patel and Tamanoi 2006). The growth arrest in case of yeast could be complemented by human Rheb showing the conservation of its functions. Overexpression of Rheb increases the cell size, while a mutant decreases its size (Patel and Tamanoi 2006). Rheb can drive cell growth in starved animals, suggesting a role for Rheb in the nutritional control of cell growth. In case of *D. discoideum,* we found the Rheb overexpressers to be slightly slow growers as compared to wild-type but with no obvious alterations in the developmental profile (data not shown). Our results show that overexpression of Rheb helps increase the cell size. The prestalk cells are slightly larger in size as compared to the prespore cells, but when we drove the expression of Rheb under the prespore-specific promoter D19, we found the cell size to increase. The multicellular structures developed visually did not show any apparent differences in the overall size of stalk and spore mass. However, in mammalian NIH 3 T3 cells, expression of constitutively active Rheb did not result in any altered phenotype (Clark *et al.* 1997). Previous studies in *Drosophila* with respect to Rheb function have shown a dramatic increase in cell size with no change in the cell number (Patel *et al.* 2003). This can be attributed to the fact that although Rheb strongly promotes G1-S progression, a corresponding extension of S and G2 phase results in overall preservation of a normal cell division rate (Saucedo *et al.* 2003). In case of *D. discoideum*, there may not be any significant changes observed in the developmental phenotypes, as development here is not associated with division like in other metazoans. Development in this case is initiated by the coming together of spatially segregated cell types and involves no cell division.

Constitutively active mutants of Rheb induce oncogenic transformation in cell culture and allow these cells to become larger in size and contain more protein than the wild-type. They show constitutive phosphorylation of the ribosomal protein S6 kinase and the eukaryotic initiation factor 4EBP1, two downstream targets of TOR. The TOR-specific inhibitor rapamycin strongly interferes with transformation induced by constitutively active Rheb, suggesting that TOR activity is essential for the oncogenic effects of mutant Rheb. The critical regulators between PKB and TOR are the TSC1/TSC2 complex where Rheb acts as a proximal activator of TOR. In addition, growth factors inhibit TSC-Rheb-GAP function, whereas hypoxia and energy depletion disinhibit the TSC-GAP activity to lower the levels of Rheb-GTP. Furthermore, withdrawal of extracellular amino acids also inactivates TORC1 signalling although it does not affect the Rheb-GTP charging, i.e. the association of GTP with Rheb, which also confers the active state of Rheb. In addition, the kinase activity of TOR polypeptide is determined by the state of Rheb nucleotide charging. However, Rheb-GTP charging does not promote Rheb-mTOR interaction. The interaction of Rheb and mTOR is necessary for the activation of TOR activity.

Acknowledgements

SS thanks CSIR, India for grants (# 37(1215)/05/EMR-II and #37/1416/10/EMR-II). PBS thanks UGC for fellowship. We acknowledge AIRF, JNU for the confocal images.

References

Aspuria PJ and Tamanoi F 2004 The Rheb family of GTP-binding proteins. *Cell. Signal.* **16** 1105–11012

Buerger C, DeVries B and Stambolic V 2006 Localization of Rheb to the endomembrane is critical for its signaling function. *Biochem. Biophys. Res. Commun.* **344** 869–880

Clark GJ, Kinch MS, Rogers-Graham K, Sebti SM, Hamilton AD and Der CJ 1997 The Ras-related protein Rheb is farnesylated and antagonizes Ras signaling and transformation. *J. Biol. Chem.* **272** 10608–10615

Early AE, Gaskell MJ, Traynor D and Williams JG 1993 Two distinct populations of prestalk cells within the tip of the migratory *Dictyostelium* slug with differing fates at culmination. *Development* **118** 353–362

Gaskell M, Watts D, Treffry T, Jermyn KA and Williams JG 1992 Immunolocalization and separation of multiple prestalk cell types in *Dictyostelium. Differentiation* **57** 171–176

Gosain A, Lohia R, Shrivastava A and Saran S 2012 Identification and characterization of peptide: N-glycanase from *Dictyostelium discoideum. BMC Biochem.* **8** 13–19

Hay N and Sonenberg N 2004 Upstream and downstream of mTOR. *Genes Dev.* **18** 1926–1945

Im E, von Lintig FC, Chen J, Zhuang S, Qui W, Chowdhury S, Worley PF, Boss GR and Pilz RB 2002 Rheb is in a high activation state and inhibits B-Raf kinase in mammalian cells. *Oncogene* **21** 6356–6365

Inoki K, Li Y, Xu T and Guan K L 2003 Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. *Genes Dev.* **17** 1829–1834

Inoki K, Kim J and Guan KL 2012 AMPK and mTOR in Cellular Energy Homeostasis and Drug Targets. *Annu. Rev. Pharmacol. Toxicol.* **52** 381–400

Jermyn KA and Williams JG 1991 An analysis of culmination in *Dictyostelium* using prestalk and stalk-specific cell autonomous markers. *Development* **111** 779–787

Jermyn K, Traynor D and Williams J 1996 The initiation of basal disc formation in *Dictyostelium discoideum* is an early event in culmination. *Development* **122** 753–760

Jiang H and Vogt PK 2008 Constitutively active Rheb induces oncogenic transformation. *Oncogene* **27** 5729–5740

Kim J, Kundu M, Viollet B and Guan KL 2011 AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat. Cell Biol.* **13** 132–143

Ma D, Bai X, Guo S and Jiang Y 2008 The switch I region of Rheb is critical for its interaction with FKBP38. *J. Biol. Chem.* **283** 25963–25970

Patel PH and Tamanoi F 2006 Using Drosophila and yeast genetics to investigate a role for the Rheb GTPase in cell growth. *Methods Enzymol.* **407** 443–454

Patel PH, Thapar N, Guo L, Martinez M, Maris J, Gau CL, Lengyel JA and Tamanoi F 2003 Drosophila Rheb GTPase is required for cell cycle progression and cell growth. *J. Cell Sci.* **116** 3601–3610

Ratner D and Borth W 1983 Comparison of differentiating *Dictyostelium discoideum* cell types separated by an improved method of density gradient centrifugation. *Exp. Cell Res.* **143** 1–13

Rojas AM, Fuentes G, Rausell A and Valencia A 2012 The Ras protein superfamily: Evolutionary tree and role of conserved amino acids. *J. Cell Biol.* **196** 189–201

Rosel D, Khurana T, Majithia A, Huang X, Bhandari R and Kimmel AR 2012 TOR complex 2 (TORC2) in *Dictyostelium* suppresses phagocytic nutrient capture independently of TORC1mediated nutrient sensing. *J. Cell Sci.* **125** 37–48

Sancak Y, Peterson TR, Shaul YD, Lindquist RA, Thoreen CC, Bar-Peled L and Sabatini DM 2008 The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science* **320** 1496–1501

Saucedo LJ, Gao X, Chiarelli DA, Li L, Pan D and Edgar BA 2003 Rheb promotes cell growth as a component of the insulin/TOR signaling network. *Nat. Cell Biol.* **5** 566–571

Stocker H, Radimerski T, Schindelholz B, Wittwer F, Belawat P, Daram P, Breuer S, Thomas G and Hafen E 2003 Rheb is an essential regulator of S6K in controlling cell growth in Drosophila. *Nat. Cell Biol.* **l5** 559–565

Tamanoi F 2011 Ras signaling in yeast. *Genes Cancer* **2** 210–215

Urano J, Ellis C, Clark GJ and Tamanoi F 2001 Characterization of Rheb functions using yeast and mammalian systems. *Methods Enzymol*. **333** 217–231

van Dam TJ, Bos JL and Snel B 2011 Evolution of the Ras-like small GTPases and their regulators. *Small GTPases* **2** 4–16

Wang X and Proud CG 2009 Nutrient control of TORC1, a cell-cycle regulator. *Trends Cell Biol.* **19** 260–267

Williams JG, Duffy KT, Lane DP, McRobbie SJ, Harwood AJ, Traynor D, Kay RR and Jermyn KA 1989 Origins of the prestalk-prespore pattern in *Dictyostelium* development. *Cell* **59** 1157–1163

Yadav RB, Burgos P, Parker AW, Iadevaia V, Proud CG, Allen RA, O'Connell JP, Jeshtadi A, Stubbs CD and Botchway SW 2013 mTOR direct interactions with Rheb-GTPase and raptor: sub-cellular localization using fluorescence lifetime imaging. *BMC Cell Biol.* **12** 14–23

MS received 14 May 2013; accepted 18 November 2013

Corresponding editor: B JAGADEESHWAR RAO