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Abstract

Understanding the mechanisms through which multicellular organisms regulate cell, organ and body growth is of relevance to developmental biology and to research on growth-related diseases such as cancer. Here we describe a new effector in growth control, the small GTPase Rheb (Ras homologue enriched in brain). Mutations in the Drosophila melanogaster Rheb gene were isolated as growth-inhibitors, whereas overexpression of Rheb promoted cell growth. Our genetic and biochemical analyses suggest that Rheb functions downstream of the tumour suppressors Tsc1 (tuberous sclerosis 1)-Tsc2 in the TOR (target of rapamycin) signalling pathway to control growth, and that a major effector of Rheb function is ribosomal S6 kinase (S6K).
Rheb is an essential regulator of S6K in controlling cell growth in Drosophila

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Understanding the mechanisms through which multicellular organisms regulate cell, organ and body growth is of relevance to developmental biology and to research on growth-related diseases such as cancer. Here we describe a new effector in growth control, the small GTPase Rheb (Ras homologue enriched in brain). Mutations in the Drosophila melanogaster Rheb gene were isolated as growth-inhibitors, whereas overexpression of Rheb promoted cell growth. Our genetic and biochemical analyses suggest that Rheb functions downstream of the tumour suppressors Tsc1 (tuberous sclerosis 1)–Tsc2 in the TOR (target of rapamycin) signalling pathway to control growth, and that a major effector of Rheb function is ribosomal S6 kinase (S6K).

A growing number of studies in genetically tractable model organisms, such as D. melanogaster, have greatly enhanced our understanding of growth regulation. From these efforts, two highly conserved signalling pathways dedicated to the control of growth have emerged, namely the insulin receptor (InR)/phosphatidylinositol-3-OH kinase (PI(3)K) and TOR pathways1,2. Recent studies have also shown that these two pathways interact, although the mechanisms by which they communicate are the subject of controversy3,4. In addition, each pathway seems to be modulated by distinct tumour suppressor genes, PTEN (phosphatase and tensin homologue deleted in chromosome 10) and TSC1–TSC2, respectively5,6. Whereas it is clear that PTEN constrains PI(3)K signalling by dephosphorylation of phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P3), the main mechanism by which TSC1 and TSC2 counteract TOR signalling remains elusive. Importantly, TSC2 possesses a putative GTPase-activating protein (GAP) domain, which has been shown to increase the intrinsic GTPase activity of the small GTPases Rap1 and Rab5 (refs 8, 9). Here, we present genetic and biochemical analyses suggesting a novel role for the small GTPase Rheb in the TOR/S6K signalling pathway.

To identify growth-regulating genes, we performed two complementarity screens for loss- and gain-of-function mutations, respectively. In the loss-of-function screen, we discovered a novel complementation group of ten alleles that impair cell and organ growth. The ethylmethane sulphonate (EMS)-induced mutations were identified on the basis of reduced head size of mosaic animals, consisting of heads largely made up of homozygous mutant cells and bodies containing heterozygous cells (compare Fig. 1a with Fig. 1b)10,11. This phenotype is reminiscent of mutations in InR signalling components. Genetic mapping of two representative alleles and subsequent testing of candidate open reading frames identified alterations in the gene CG1081 in seven alleles. CG1081 encodes a small GTPase most closely related to mammalian Rheb12 (Supplementary Information, Fig. S1a). Therefore, we named this complementation group Rheb.

The gain-of-function screen for genes that stimulate growth when overexpressed resulted in the identification of an EP element in the Rheb locus (EP 50.084). EP-mediated overexpression of Rheb in the developing eye substantially increased eye size (Fig. 1c). We generated six additional Rheb loss-of-function alleles by imprecise excision of EP 50.084. Whereas all combinations of the EMS-induced Rheb alleles were lethal, some hetero-allelic combinations of EMS-induced alleles and EP excision alleles were viable and resulted in flies of reduced size (Supplementary Information, Fig. S1b). The size reduction was caused by a decrease in cell number (3–11%), as well as in cell size (9–14% in wing cells, more than 20% in eye cells as judged by ommatidial size). In addition, the small flies eclosed with a delay of at least one day and the females had rudimentary ovaries and were sterile (Fig. 1d). Thus, the surviving Rheb mutant flies display all the hallmarks of impaired InR signalling activity, resembling flies lacking the insulin-receptor substrate (IRS) protein Chico10.

A more severe reduction in Rheb function (in heteroallelic combinations of Rheb mutations) was lethal at late larval or early pupal stages. Mutant larvae and pupae were consistently smaller (Fig. 1e, f), although the phenotype was variable. Interestingly, the size reduction was more pronounced in the endoreplicative larval tissue than in the imaginal discs (Fig. 1g–i), similarly to the larval phenotype of TOR mutants13,14. Staining of DNA in salivary glands and fat body cells demonstrated a severe deficit in endoreplication (Fig. 1j).

The behaviour of Rheb mutant cells was studied during development by means of mitotic recombination. Clones of cells homozygous for EMS-induced Rheb alleles grew poorly and were consistently smaller than their corresponding sister clones (Fig. 2a). When provided with a proliferative advantage (by means of the Min technique), Rheb mutant cells still failed to cover large regions of the

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imaginal discs. Instead, the resulting clones typically displayed elongated shapes with thin extensions (Supplementary Information, Fig. S2a). A possible explanation for this unusual phenotype may reside in their attempt to minimise contact with other mutant cells. To our knowledge, this phenomenon has not been previously described in the context of growth-regulating genes. Despite this abnormal behaviour, \textit{Rheb} mutant cells differentiate properly into adult structures. For example, analysis of clones in the adult eye revealed the presence of extremely small photoreceptor cells of otherwise normal structure and arrangement in the mutant tissue (Fig. 2b). The size reduction phenotype is strictly cell-autonomous. Taken together, the characterisation of the mutant phenotypes demonstrates that \textit{Rheb} is required for proper growth regulation in a cell-autonomous manner.

Next, we examined whether overexpression of \textit{Rheb} is sufficient to promote growth. The effect of overexpressing \textit{Rheb} during development through the use of the EP 50.084 line and two independent UAS-\textit{Rheb} lines was monitored in marked clones in imaginal discs and in the adult eye. All the lines yielded qualitatively similar results, with the EP line consistently showing the strongest effects. Clones overexpressing \textit{Rheb} in the wing imaginal disc attained a substantially larger size when compared with control clones (Fig. 2c). This enlargement is caused by a significant increase in cell size (a 48% increase in area covered per cell). In contrast, the cell doubling time remained unchanged in cells expressing \textit{Rheb} versus control cells (10.5 h versus 10.8 h). Consistent with the size effect in the imaginal discs, cells expressing \textit{Rheb} in differentiating cells posterior to the morphogenetic furrow (under the control of GMR regulatory sequences) resulted in enlarged but fully differentiated photoreceptor cells (a 66% size increase of the rhabdomeres; Fig. 2d). As in the case of the loss-of-function clones, the size alteration was cell-autonomous. Thus, \textit{Rheb} is sufficient to promote cellular growth.

As both InR and TOR signalling have been implicated in the response to nutrient availability\textsuperscript{15,16}, we asked whether overexpression of \textit{Rheb} would promote growth even under starvation conditions. It

**Figure 1** Growth defects caused by \textit{Rheb} mutations. (a–c) Phenotypes that resulted in the identification of \textit{Rheb} alleles. Shown are SEMs of female fly eyes taken at the same magnification. EMS-induced \textit{Rheb} loss-of-function alleles were identified on the basis of the pinhead phenotype in mosaic animals with largely homozygous mutant heads, as shown in a. A comparison with a control eye, as shown in b, demonstrates the marked size reduction. An EP-element driving \textit{Rheb} expression was isolated as a gain-of-function mutation promoting overgrowth in the eye, as shown in c. (d) Ovaries of viable \textit{Rheb}\textsuperscript{271}/\textit{Rheb}\textsuperscript{241} females (bottom) are markedly reduced in size when compared with control ovaries (top). (e, f) Phenotype of a pupal lethal heteroallelic combination (\textit{Rheb}\textsuperscript{241}/\textit{Rheb}\textsuperscript{26.2}). The size range of mutant larvae (e, middle and right) is shown in comparison with a control larva (left). Whereas the smaller mutant larvae die, the larger mutant larvae arrest development at the early pupal stage. A corresponding pupa (f, right) is clearly smaller than the control pupa (left). (g–j) Size defects of imaginal discs and salivary glands in larvae of a pupal lethal heteroallelic combination (\textit{Rheb}\textsuperscript{241}/\textit{Rheb}\textsuperscript{26.2}). Whereas the size reduction of mutant imaginal discs is roughly proportional to the larval size (g, control; h, mutant), the salivary glands are more severely reduced (compare j to control gland in i). DAPI staining (blue) shows a strong endoreplication deficit in \textit{Rheb} mutant salivary glands. Membranes are stained with an anti-lin7 antibody (red).
Figure 2 Clonal analysis of Rheb function. (a) Loss-of-function clones in the wing imaginal disc are consistently smaller than their corresponding twin clones. A clone of cells homozygous mutant for Rheb<sup>2D1</sup> (marked by the absence of GFP) is only about half the size of its twin spot (bright green staining). Nuclei are labelled by DAPI staining (shown in red). The reduced clone size is accounted for by fewer and smaller cells (<i>n</i> = 10, <i>P</i> = 0.00038 for cell number, <i>P</i> = 0.00126 for cell size). (b) Tangential sections through compound eyes reveal that cells homozygous mutant for Rheb<sup>3M2</sup> (marked by the lack of pigments) are able to differentiate into the various cell types of the eye. The size of the mutant cells, however, is greatly reduced (as reflected by a 72% reduction in rhabdomere area in tangential sections). A mosaic ommatidium containing a single mutant photoreceptor cell (inset, asterisk) demonstrates the cell-autonomy of the size reduction. (c) Flp-out clones of Rheb-expressing cells in the wing imaginal disc attain a significantly larger size. Clones 40 h after induction and marked by GFP expression are shown (top: control, bottom: expressing Rheb). The increase in clonal area is caused by larger cells (<i>n</i> = 20, <i>P</i> = 0.00002), whereas the number of cells is not significantly altered (<i>P</i> = 0.087). (d) Expression of Rheb during eye development results in an enlargement of photoreceptor cells. Cells expressing Rheb under GMR-control are marked by the absence of pigmentation. Cell-autonomy of the size increase is demonstrated by a single Rheb-expressing photoreceptor cell (asterisk) in a mosaic ommatidium (inset). (e–h) The effects of Rheb expression in endoreplicative larval tissues under normal conditions (e, g) and under amino-acid deprivation (f, h). Cells expressing Rheb (labelled with GFP, arrows in lower panels) under normal nutrient availability in salivary glands (e) and fat body cells (g) display only a mild size increase. In larvae starved for amino acids, however, Rheb expression exerts strong effects on both cell size and DNA ploidy. In salivary glands (f), Rheb-expressing cells display a several fold increase in size and contain much more DNA (stained with DAPI, lower panels) than non-expressing neighbouring cells, but they do not reach normal size and ploidy. In the fat body cells (h), expression of Rheb reverses the starvation phenotype completely. Size, DNA content, and appearance (amount of vesicles) of these cells are indistinguishable from non-starved cells. Membranes are visualised with an anti-lin7 antibody staining (red). Separate DAPI staining is shown in lower panels (blue).
has been shown that depriving larvae of amino acids blocks endoreduplication of the larval tissues, but that this can be overcome by expression of Dp110/PI(3)K16. Rheb was expressed in small clones of cells in the salivary glands and in the fat body. Under normal food conditions, only a very subtle increase in cell size was observed (Fig. 2e, h). Despite the lack of amino acids, larval cells expressing Rheb reached a normal size in the fat body, and the size and endoreduplication deficits were significantly alleviated in the salivary glands. We conclude that Rheb is sufficient to counteract the effects of amino-acid deprivation and thus may function in amino-acid sensing.

Given the similarities between Rheb and mutants in the InR and TOR signalling pathways, it is conceivable that Rheb represents a novel component of one of these growth control pathways. To test this possibility, a detailed epistasis analysis was performed. First, we tested whether the negative regulators of InR and TOR signalling — PTEN, Rheb, Tsc1, and Tsc2, respectively — could counteract the effects of Rheb overexpression. All overexpression experiments were performed in the eye using the GMR-Gal4 driver line. Expression of either PTEN or Tsc1–Tsc2 alone resulted in a very similar size reduction of the ommatidia (Fig. 3b, c) when compared with control ommatidia (Fig. 3a). However, whereas expression of PTEN had no influence on the increase in ommatidial size caused by Rheb overexpression (Fig. 3g),

The effects of Rheb overexpression are dominantly alleviated by TOR2L1 (k).

Figure 3  Epistatic relationship of Rheb with the InR/dPI(3)K and the TOR signalling pathways. (a–l) SEMs showing ommatидial size and shape of female flies. (a) Control (b) GMR-Gal4/+; UAS-PTEN+ (c) GMR-Gal4+; UAS-Tsc1 UAS-Tsc2+ (d) PKB+/PKB+ (e) S6K1/S6K2+ (f) GMR-Gal4+; EP50.084/+ (g) GMR-Gal4+; EP50.084/UAS-PTEN (h) GMR-Gal4+ (i) GMR-Gal4+; EP50.084 PKB+/PKB+ (j) GMR-Gal4+; S6K1+ EP50.084/S6K2+ (k) GMR-Gal4; TOR2L1+ EP50.084/S6K2+ (l) GMR-Gal4+; S6K1+ EP50.084/S6K2+ Ommatidial size relative to the control is indicated in each panel. Expression of Rheb under GMR-Gal4 control results in large and disorganised ommatidia (f). This phenotype can be suppressed by co-expression of Tsc1–Tsc2 (h), but not by PTEN (g). The enlarged ommatidial size is still evident in a PKB mutant background (l), but is neutralised in flies lacking S6K function (j).
co-expression of Tsc1–Tsc2 resulted in ommatidia of approximately wild-type size (Fig. 3h), indicating that the activities of Rheb and Tsc1–Tsc2 can counteract each other. Next, the enlarged ommatidial phenotype of GMR-Rheb was assayed in a number of mutant backgrounds. Reducing the activity of Drosophila protein kinase B (PKB) had no effect on the ommatidial size (Fig. 3i). Similar results were obtained with hypomorphic mutations in InR and Dp110, respectively (data not shown). In contrast, ommatidial size was dominantly reduced by a mutation in TOR (TOR2L1; Fig. 3k), and a suppression to wild-type size was observed in a S6K mutant background (Fig. 3j). Thus, the Rheb overexpression phenotype is dependent on TOR and S6K function, but is independent of InR signal strength. Finally, we analysed the behaviour of Rheb PTEN and Rheb Tsc1 double mutants. The phenotypic consequences were assayed in mosaic animals using the ey-Flp method. As expected, the Rheb PTEN double-mutant tissue clearly displayed a Rheb phenotype (Fig. 3q). The Rheb Tsc1 mutant tissue also resembled Rheb single mutants (Fig. 3r), indicating that Rheb is epistatic over Tsc1.

Complete loss of Tsc1 function results in larval lethality. Importantly, we found that a simultaneous reduction of Rheb function was sufficient to restore viability. The emerging double-mutant flies displayed a weak Rheb hypomorphic phenotype (a moderate size reduction; Fig. 3s). These findings suggest that the major consequence of a lack of Tsc1 is overactivation of Rheb.

Our genetic analysis indicated that Rheb regulates S6K through TOR. Therefore, we tested whether S6K activity is dependent on Rheb function. Larval extracts of various heteroallelic Rheb combinations were subjected to S6K and PKB kinase assays. Indeed, S6K activity was significantly reduced in all combinations without any apparent effect on S6K protein levels (Fig. 3a). PKB activity, however, was consistently increased (Fig. 4b). This is in agreement with the hypothesis that S6K is an essential component of a negative feedback loop regulating InR signalling. Conversely, ubiquitous expression of Rheb resulted in an increase in S6K activity (Fig. 4c) and a concomitant decrease in PKB activity (Fig. 4d). The stimulation of S6K activity by Rheb was also observed after amino-acid deprivation (Fig. 4e). Thus, Rheb is both necessary and sufficient for S6K activation.

Although Rheb is essential for S6K activity, and the overgrowth phenotype elicited by Rheb overexpression depends on S6K, regulation of S6K is clearly not the only effect of Rheb activity. Whereas flies lacking S6K function are semi-viable (exhibiting a severe delay in development and a reduced body size), loss of Rheb is lethal. Moreover, reduction of Rheb activity results in a decrease in cell number and cell size (as opposed to S6K mutants, which only affect cell size). Finally, the characteristic shape of S6K mutant cell clones suggests that Rheb has other functions in addition to growth control.

Two models of Rheb activity can be envisaged: first, Rheb could function in the TOR signalling pathway directly downstream of and negatively regulated by Tsc1–Tsc2 (Supplementary Information, Fig. S3a). Second, Rheb might be a component of an independent pathway that impinges on S6K (Supplementary Information, Fig. S3b). In the latter model, the TOR signalling pathway and the putative parallel pathway would both be necessary for the full activation of S6K. This could explain why impairing the activity of one pathway interferes with the consequences of overactivating the other. Nevertheless, we favour the former model because Rheb mutants show
striking similarities with TOR signalling defects and because of the intimate genetic interactions of *Rheb* with *Tsc1*–*Tsc2*. A particularly attractive hypothesis implicates *Tsc2* as the GAP of *Rheb*. Indeed, in an accompanying paper, Zhang *et al.* provide evidence that *Tsc2* is the GAP for *Rheb* in *Drosophila* (this issue), and the same conclusion has been derived from studies on the mammalian homologues of *Drosophila* *Tsc2* and *Rheb* (F.J.T. Zwartkruis, J.L. Bos and G.T., personal communication).

Interestingly, loss of *rbi* function in the fission yeast *Schizosaccharomyces pombe* results in a growth arrest phenotype that is very similar to that of nitrogen-starved cells. Thus, the function of *Rheb* in growth regulation in response to nutrients (amino acids) may have been conserved during evolution. Furthermore, the fact that impaired *rbi* function is sufficient to suppress the phenotypic consequences of loss of *PTEN* and *TSC1*–*TSC2* suggests that *Rheb* might be a suitable target for therapeutic intervention in a wide range of tumours.

**METHODS**

**Genetics.** EMS-induced *Rheb* alleles were isolated in an *ey-Flp* mosaic screen that will be described elsewhere. Two alleles, *Rheb*44.1 and *Rheb*56, were mapped genetically with respect to visible markers. A subsequent fine mapping step placed the mutations between the *P*-element insertions EP7947 (83A3) and I(3)1C2 (83C1-2) in a candidate region of 312.5 kb. Eight polymorphisms distributed over the candidate region were used to establish a high-resolution SNP map. The candidate region could be narrowed down to 60 kb by mapping the recombination sites between the *Rheb* alleles and the two P elements using SNP detection by DHPLC. Candidate open reading frames in this interval were amplified by PCR from heterozygous flies and tested for polymorphisms by DHPLC. Amplified fragments of *CG1081* displayed various polymorphisms and were subsequently sequenced.

The EP line 50.084 was identified among 10,000 novel insertions of a double-headed EP element. It is inserted six nucleotides downstream of the 5′ end of the first exon of transcript CG1081-RA (GaCfly database). The EP50.084 chromosome carries a closely linked lethal factor that cannot be reversed by mobilization of the EP element. The UAS sites at the 5′ end of EP50.084 were excised by Cre-loxP-mediated recombination to yield a single-headed EP element capable of driving *Rheb*. EP50.084 was remodelled by crossing in the transposase source A2-3 to generate impocrine excision alleles. At least 83 independent revertants of the dominant yellow marker were recovered; six of them failed to complement the *Rheb* alleles. The two impocrine excision alleles that yielded viable combinations with some EMS-alleles (*Rheb*44.1 and *Rheb*56) retained partial sequences of the EP element (0.6 and 1.1 kb, respectively) without deleting any flanking genomic sequences.

The following transgenes and mutations were used for interaction studies: *GMR-Gal4* (ref. 28), UAS-PTEN (ref. 29), UAS-Tsc1, UAS-ACT (ref. 17), PPK (ref. 29), PKB (ref. 30), *TOR*2L1 (ref. 13), *S6K1*2L (ref. 24), *PTEN*2L17 (ref. 31), *Tsc1*2L2 and *Tsc1*1L2 (H.S. and E.H., unpublished observations).

To generate loss-of-function clones, we used the lines *y* > *hs-flp*, FRT82 *Ubi-GFP* M1/TM6B for clones in the imaginal discs, and *y* > *hs-flp*, FRT82 w*+* M/TM6B for clones in the adult eye. Clones were induced in 48–72-h-old larvae by a 45-min heat-shock at 37 °C. To circumvent potential side effects caused by second hits, at least three different EMS-induced *Rheb* alleles were tested in each experiment. The results were always qualitatively similar.

Overexpression clones were generated by means of the ‘FLP-out’ technique using the lines *y* > hs-flp; *Act>CD2>G4d UAS-GFPHs* M/TM6B (ref. 32) and *y* > *hs-flp; GFPVn>*G4d* (ref. 33), respectively. FLP-out clones were induced either in 80-h-old larvae by a 13-min heat-shock at 34 °C (*Act>CD2>G4d*), or in 24–48-h-old larvae by a 1-h heat-shock at 37 °C (*GMRVn>*G4d*). To achieve overexpression in the endoreplicative larval tissues, spontaneous FLP-out events using *y* > hs-flp; *Act>CD2>G4d UAS-GFPHs* M/TM6B without heat-shock application were recovered. All the overexpression experiments were performed using EP50.084 and two UAS-Rheb lines (individually or in combination). The severity of the resulting phenotypes was always in accordance with the order EP50.084 > UAS-Rheb13.1 > UAS-Rheb14.2 > UAS-Rheb14.3, presumably reflecting different expression levels. To deprive larvae of dietary amino acids, 60-h-old larvae were incubated in 20% sucrose solution.

Eye-specific clones were generated using the ey-Flp system, as previously described. For the double-mutant analysis of *PTEN Rheb* clones, the ey-Flp systems for 2L and 3R were combined to induce clones simultaneously for FRT40 *PTEN*2L117 and FRT82 *Rheb*. To study the effects of *Rheb Tsc1* double mutants, recombinant chromosomes were generated with various alleles. *Gal4* under control of a heat-shock promoter (*hs-Gal4*) was used to ubiquitously overexpress *Rheb*. Second-instar larvae were transferred to tubes containing fresh squashed fly food and placed in a waterbath at 37 °C for 45 min. Then larvae were allowed to recover at room temperature for the indicated times before analysis in kinase assays.

**Extraction of larvae, kinase assays and western blotting.** Larvae were extracted essentially as described, however with a modified extraction buffer (120 mM sodium chloride, 50 mM Tris-Cl at pH 7.0, 20 mM sodium fluoride, 1 mM benzamidine, 1 mM EDTA, 6 mM EGTA, 15 mM Na4P2O7-10H2O and 1% NP-40; the following reagents were added shortly before use to the indicated concentrations): 1/5 volume Complete Mini protease inhibitor cocktail (Roche Diagnostics, Rotkreuz, Switzerland) prepared as a 5× stock in extraction buffer without the reagents listed hereafter, 2 mM AEBSF (Pefabloc SC (Roche)), 30 mM para-nitrophenylphosphosphate, 30 mM β-glycerolphosphate, 4 mM leupeptin, 2 μM aprotinin, 4 μM pepstatin and 100 μM phenylmethylsulphonyl fluoride).

Kinase activity assays of either *S6K* or *PKB* were performed as described. For immunoblotting, the following antibodies were used at the indicated dilutions: *S6K* D-20 antibody at 1:200 (ref. 24), PKB antibody (ref. 34) at 1:2000, S6 antibody, 1:2000 (ref. 25), and antibodies to caspase-3, -9, -12, and -7 (Cell Signaling Technology). EGFP being tagged with Flag resulted in a strong overexpression of the antibody to Flag. The following antibodies were used at 1:1000 and β-tubulin antibody (Sigma, St Louis, MO) at 1:1000. HRP-conjugated secondary antibodies (Dako A/S, Glostrup, Denmark) were diluted 1:2000. Signals were detected using ECL western blotting detection reagents (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

**Note:** Supplementary Information is available on the *Nature Cell Biology* website.

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**COMPETING FINANCIAL INTERESTS**

The authors declare that they have no competing financial interests.

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Figure S1. **Mutations in the highly conserved dRheb protein result in growth impairment.**

a. At the amino acid level, dRheb shares 63% identity and 81% similarity with human Rheb, and 51% identity and 72% similarity with S. pombe rhb1p, respectively. The effector domain (amino acids 34-42 in dRheb) is identical in human and Drosophila Rheb. The carboxy-terminal part is less well conserved. All proteins contain a farnesylation sequence at the very carboxy-terminus. The alterations resulting from the EMS-induced dRheb mutations are: dRheb<sup>3M2</sup>: translation initiation codon mutated to ACG, dRheb<sup>5O1</sup>: I<sub>68</sub>M, dRheb<sup>7A1</sup>: V<sub>71</sub>K, dRheb<sup>4L1</sup>: K<sub>101</sub>Stop, dRheb<sup>2D1</sup>: two nucleotide exchanges in the 5' UTR, dRheb<sup>2G5</sup>: a combination of a deletion of eleven nucleotides and an insertion of six nucleotides in the second intron, generating a putative novel splice acceptor site, dRheb<sup>1T1</sup>: splice site mutation predicted to result in a protein consisting of the amino-terminal 16 amino acids followed by DEFSQKVPEALTLLLLALV.

b. Quantification of the size defects observed in heteroallelic combinations of dRheb alleles. The observed size reduction is due to fewer and smaller cells in the eyes and in the wings. All the reductions, with the exception of the reduced wing cell number in the genotype dRheb<sup>2O5</sup>/dRheb<sup>44.1</sup> (p=0.16), are statistically significant (p≤0.01).
Figure S2. **Unusual behaviour of cells lacking or overexpressing dRheb.** a, Even when provided with a growth advantage (by means of the Minute technique), dRheb mutant cells fail to generate large clones. Instead, these clones are characterised by extended thin branches (a'). Smaller clones that were induced later already display an elongated shape (a). b, Clones of dRheb expressing cells have a higher tendency to fuse. The single clone on the left touches a larger group of cells probably consisting of two fused clones. Occasionally, fusions of multiple clones can be observed (b').
Figure S3  Alternative positions of dRheb in the growth regulatory network. Schematic representation of the dInr/dPI3K and dTOR signalling pathways. The activity of dS6K is under the control of dPDK1 and dTOR. Two links between the dInr/dPI3K and the dTOR pathways have recently been described. dPKB can negatively regulate dTsc1/2 function by phosphorylating dTsc2, and dS6K negatively regulates dPKB activity (although the target of this negative feedback remains unknown). In our favoured model, dRheb is negatively regulated by the dTsc1/2 complex (possibly by the GAP activity of dTsc2) and positively acts on dTOR function. Alternatively, dRheb may function in a pathway parallel to dTsc1/2-dTOR whose activity is essential for full activation of dS6K. The more severe (complex) phenotypes caused by mutations in dPKB, dTOR, or dRheb as compared to mutations in dS6K suggest that these proteins have additional as yet unknown growth relevant targets (block arrows).