Increased Rheb-TOR signaling enhances sensitivity of the whole organism to oxidative stress

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Accepted 3 August 2006
Journal of Cell Science 119, 4285-4292 Published by The Company of Biologists 2006
doi:10.1242/jcs.03199

Summary

The accumulation of free radical damage to an organism over its lifespan can cause premature aging and disease including cancer, atherosclerosis and neurodegenerative disorders. The well-conserved Rheb–Target-of-rapamycin (TOR)–S6-kinase (S6K) signaling pathway regulates several cellular processes and has been shown to influence lifespan and diseases such as cancer and neurodegenerative disorders. Using adult *Drosophila*, we describe for the first time in metazoans that TOR activity can influence the stress response. We find that mildly increasing systemic Rheb-TOR-S6K signaling sensitizes the whole organism to oxidative stress and promotes senescence of locomotor activity with age. Furthermore, we find that S6K is required for increased Rheb-TOR signaling to sensitize the whole organism to oxidative stress and promote the senescence of locomotor activity. Interestingly, we also find that increasing Rheb-TOR signaling in muscle can increase the sensitivity of adults to oxidative stress. These data imply that pathological situations that increase TOR activity might perturb the ability of the whole organism to cope with stress causing disease progression and aging.

Key words: Rheb, TOR, S6K, Oxidative stress, Aging

Introduction

The stress endured by an organism over a lifetime can affect its health and viability. In particular, oxidative damage to biological macromolecules is thought to cause premature aging and disease, including cancer, atherosclerosis and neurodegenerative disorders (Beckman and Ames, 1998). A variety of mechanisms exists to protect an organism from stress including antioxidant enzymes, protein re-folding or degradation, DNA repair and stress-sensing pathways that effect gene expression of stress-defense genes (Finkel and Holbrook, 2000).

In eukaryotes, the target-of-rapamycin (TOR) signaling pathway regulates several processes including cell growth and protein translation (Aspuria and Tamanoi, 2004; Inoki et al., 2005b; Pan et al., 2004). TOR kinase activity is thought to stimulate protein synthesis by activation of S6 kinase (S6K) and repression of the translational inhibitor, 4E-BP. TOR proteins exist in multi-protein complexes and bind the small GTPase Rheb; Rheb proteins have been shown to stimulate TOR kinase activity (Long et al., 2005; Smith et al., 2005; Urano et al., 2005). In *Drosophila*, we as well as others have shown that *Rheb* can stimulate cell growth through TOR (Patel et al., 2003; Saucedo et al., 2003; Stocker et al., 2003). Furthermore, Tsc2, which functions as a GTPase-activating protein (GAP) when in complex with Tsc1, can negatively regulate Rheb-TOR signaling by stimulating the hydrolysis of GTP bound to Rheb, to GDP (Castro et al., 2003; Garami et al., 2003; Inoki et al., 2003; Tee et al., 2003; Zhang et al., 2003) and inhibit cell growth (Gao and Pan, 2001; Inoki et al., 2002; Potter et al., 2001; Tapon et al., 2001). However, TOR proteins also play important roles in the regulation of ribosome biogenesis, lifespan and in a number of age-related diseases (such as cancer and neurodegenerative disorders) (Inoki et al., 2005a; Sarbassov dos et al., 2005). Since evidence for the contribution of stress to aging and age-related disease is growing, the influence of TOR signaling on the response of the whole organism to stress needs to be investigated.

Here we use the genetically tractable organism *Drosophila* to examine the effect of the Rheb-TOR-S6K signaling on the stress response. *Drosophila* has proven to be a useful system to assess the effects of various signaling pathways on stress (Clancy et al., 2001; Hwangbo et al., 2004; Simon et al., 2003; Wang et al., 2003), because flies can be readily exposed to stress and the consequences of the exposure can be tested with a large number of animals over a short period of time. We find that adult flies with increased Rheb-TOR signaling through S6K are sensitive to oxidative stress. Similarly, we find that alteration of Rheb-TOR and S6K signaling affects the starvation response, suggesting that increased TOR signaling may affect the response to various forms of stress. Further, we find that increasing Rheb-TOR signaling in muscle sensitizes flies to oxidative stress and increasing Rheb-TOR signaling through S6K in the whole organism results in early senescence of locomotor activity. These data support a role for Rheb-TOR signaling in aging and age-related disease. Since levels of Rheb-TOR signaling are increased by elevated insulin levels, nutrient availability as well as in several disease states, these situations may perturb the normal stress response.

Results

Mild overexpression of *Rheb* stimulates TOR activity in adult flies

Overexpression of *Rheb* in *Drosophila* during development

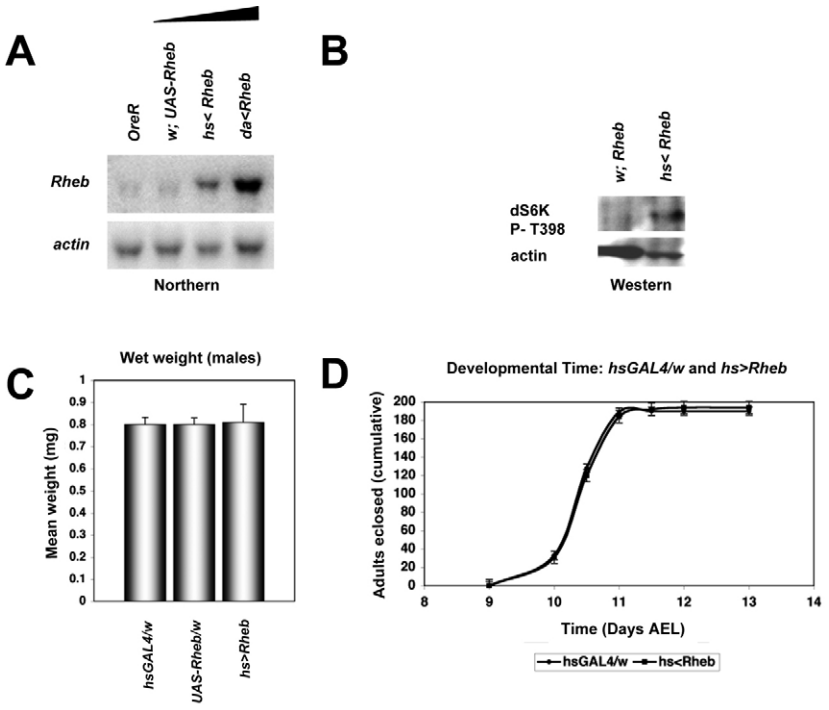

Fig. 1. Weak overexpression of *Rheb* stimulates TOR activity in adults. (A) Northern analysis of adult flies overexpressing *Rheb* with *hs-GAL4* (*hs>Rheb*) and *da-GAL4* (*da>Rheb*) shows increased *Rheb* transcript levels. (B) Western analysis of adults overexpressing *Rheb* with *hs-GAL4* shows increased phosphorylation of T398 of S6K. Flies overexpressing *Rheb* at low levels develop into adult flies with normal body size and weight (wet) (P=0.3456, NS) (C) at a similar rate as control flies (P=0.3998, NS) (D). Error bars represent the s.d. Statistical comparison (T-test): all P values are based on comparison of the overexpressor with control (*hsGAL4/w*).

results in increased cell growth and stimulation of TOR activity reflected by the increase of T398 phosphorylation of its downstream effector S6K, a positive regulator of protein synthesis (Patel et al., 2003; Saucedo et al., 2003; Stocker et al., 2003). We used the *GAL4/UAS* system (Brand and Perrimon, 1993) to ubiquitously overexpress *Rheb* in adult flies to examine the stress response of adult flies with increased Rheb-TOR activity. To generate adults overexpressing *Rheb* (*hs>Rheb* and *da>Rheb*), we crossed flies carrying the *UAS-Rheb* transgene (Patel et al., 2003) to flies carrying either *hs-* or *da-GAL4*, which allow overexpression at low and high levels, respectively. Northern analysis of total RNA from 5-day-old adults reveals that *Rheb* is overexpressed at least fourfold with *hs-GAL4* and 19-fold with *da-GAL4* at 25°C (Fig. 1A). Overexpression of *Rheb* with *hs-GAL4* results in phosphorylation of T398 of S6K (Fig. 1B) suggesting that low levels of *Rheb* overexpression can promote TOR signaling in adults. Flies overexpressing *Rheb* with *hs-GAL4* seem fit overall, as they show normal body size and weight (Fig. 1C) and display normal developmental times (Fig. 1D), thus we used these flies for the stress experiments described below.

Mild overexpression of *Rheb* sensitizes adult flies to oxidative stress

To test the effects of oxidative stress on flies with increased Rheb-TOR activity, we administered daily oxidative agents in 5% sucrose/PBS for 6 hours *hs>Rheb* to adult flies and then returned these flies to normal growth medium. As a control, each genotype was similarly fed 5% sucrose/PBS alone for 6 hours before returning them to normal growth medium. While all genotypes survive daily 6-hour feedings of the 5% sucrose/PBS diet (Fig. 2A,B), we found *hs>Rheb* flies to be sensitive to 5% sucrose/PBS containing 5% H_2O_2 (Fig. 2A). This increased sensitivity of *hs>Rheb* flies to H_2O_2 was also observed with continuous exposure to 5% H_2O_2 in 5% sucrose/PBS (data not shown). Post-mortem examination of *hs>Rheb* flies fed 5% H_2O_2 revealed necrotic gut tissue probably owing to activity of ingested H_2O_2 suggesting that mortality is due to toxicity of the oxidative agent and not due to starvation by avoidance of 5% sucrose/PBS containing 5% H_2O_2. Thus mild overexpression of *Rheb* appears to sensitize adult flies to oxidative stress. A similar sensitivity to oxidative stress can be observed in *hs>Rheb* flies fed yet another oxidative agent, 20 mM paraquat (methyl viologen) (Fig. 2B). By contrast, *hs>Rheb* flies were found to be as sensitive to 25 mg/ml G418 as control flies, suggesting that the effects of increased Rheb-TOR activity increases sensitivity to oxidative stress rather than to all toxic compounds in general (Fig. 2C).

Increased TOR and S6K signaling sensitizes flies to oxidative stress

Since flies overexpressing *Rheb* are sensitive to oxidative stress, we tested whether flies with increased TOR or S6K activity displayed a similar sensitivity. We found that overexpression of wild-type *TOR* with *hs-GAL4 (hs>TOR)* sensitized flies to oxidative stress (Fig. 3A). We were unable to ubiquitously overexpress wild-type TOR at higher levels in adults with the stronger GAL4 drivers, *da-* and *tub-GAL4*; *da>TOR* and *tub>TOR* flies arrest in growth during larval development (data not shown) similarly to TOR mutants (Oldham et al., 2000; Zhang et al., 2000).

Similarly, we found that flies overexpressing a constitutively active form of S6K ($S6K^{STDETE}$) (Barcelo and Stewart, 2002) with *da-GAL4* (*da>*$S6K^{STDETE}$) were sensitive to oxidative stress (Fig. 3A). The $S6K^{STDETE}$ variant mimics an active, hyperphosphorylated form of S6K (Barcelo and Stewart, 2002). Since strong sensitivity to oxidative stress was observed in flies overexpressing *Rheb* or $S6K^{STDETE}$, increased TOR activity might sensitize flies to oxidative stress by increasing S6K activity.

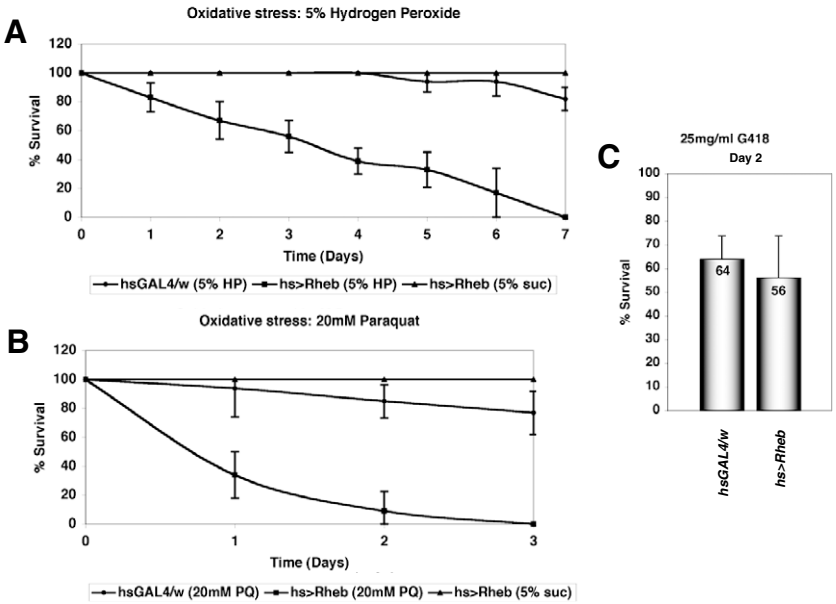

Fig. 2. Mild overexpression of *Rheb* with *hs-GAL4* (*hs>Rheb*) sensitizes adult flies to oxidative stress. Like control (*hsGAL4/w*) flies, *hs>Rheb* flies are not sensitive to daily feeding of 5% sucrose/PBS for 6 hours. However, *hs>Rheb* flies are sensitive to 5% H_2O_2 (mean survival is 33% after 5 days; n=166, P=0.0028) (A) as well as to another oxidative agent, paraquat (mean survival is 9% after 2 days; n=160, P=0.0006) (B). This mortality was not due to a general sensitivity of *hs>Rheb* flies to toxic compounds because these flies were found to be as sensitive to 25 mg/ml G418 as control flies (P=0.1193, NS). The mean survival on 25 mg/ml G418 is 64% for *hsGAL4/w* flies and 56% for *hs>Rheb* flies after 2 days (C). Error bars represent the s.d. Statistical comparison (T-test): all P values are based on comparison of the overexpressor with control.

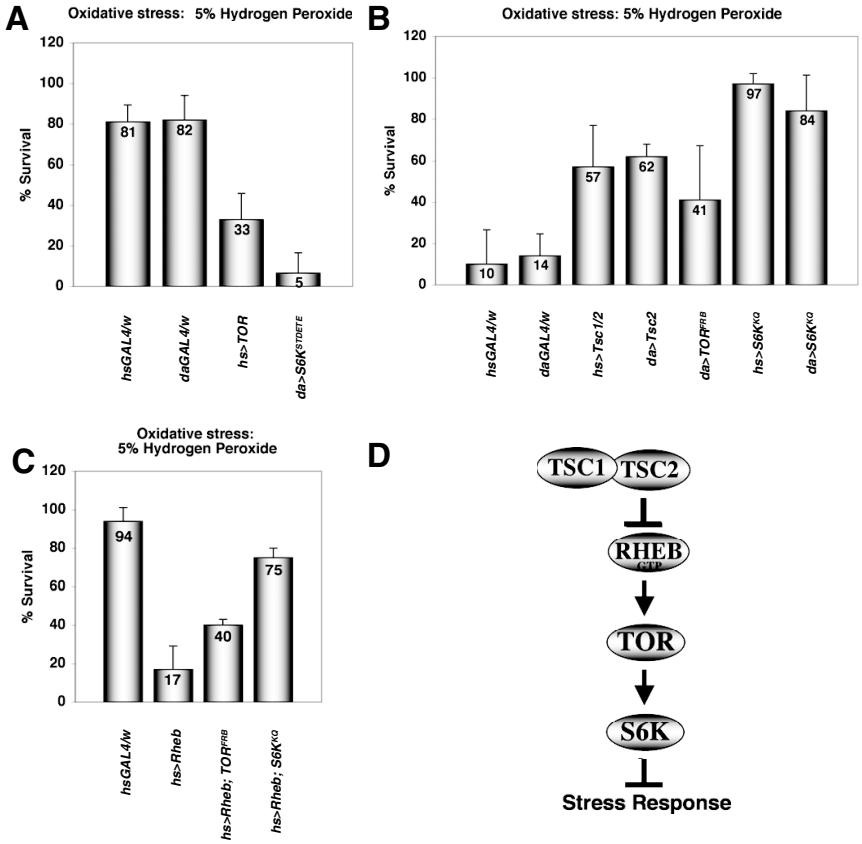

Fig. 3. Altering Rheb-TOR-S6K signaling can influence the response of adult flies to oxidative stress. Although increasing Rheb-TOR-S6K signaling sensitizes flies to oxidative stress (A), decreasing signaling through this pathway provides resistance to oxidative stress (B). Furthermore, Rheb-TOR signaling requires S6K to confer sensitivity to the whole organism to oxidative stress (C). The mean survival 24 hours after start of treatment with 5% H_2O_2 in 5% sucrose/PBS is 81% for *hsGAL4/w* flies (control) and 82% for *daGAL4/w* flies (control), but was only 33% for *hs>TOR* flies (n=117, P=0.0061) and 5% for *da>S6K*^{STDETE} flies (n=117, P=0.0014) (A). The mean survival 36 hours after start of treatment with 5% H_2O_2 in 5% sucrose/PBS is 57% for *hs>Tsc1/2* flies (n=129, P=0.0169), 62% for *da>Tsc2* flies (n=126, P=0.0059), 41% for *da>TOR*^{FRB} flies (n=106, P=0.3194), 97% for *hs>S6K*^{KQ} flies (n=117, P=0.0030) and 84% for *da>S6K*^{KQ} flies (n=117, P=0.0401), it was however, only 10% for *hsGAL4/w* flies (control) and 14% for *daGAL4/w* flies (control) (B). Overexpression of a dominant-negative form of *TOR*^{FRB} can partially rescue the sensitivity of flies overexpressing *Rheb* with *hs-GAL4* (*hs>Rheb*) (C). Furthermore overexpression of a dominant-negative form of *S6K*^{KQ} can fully rescue the sensitivity of flies overexpressing *Rheb* (P=0.1835, NS) (C). The mean survival after 6 days is 94% for *hsGAL4/w* flies (control), 17% for *hs>Rheb* flies, 40% for *hs>Rheb; TOR*^{FRB} flies and 75% for *hs>Rheb; S6K*^{KQ} flies (C). Error bars represent the s.d. Statistical comparison (T-test): all P values are based on comparison of the overexpressor with controls (*hsGAL4/w* and *daGAL4/w*). (D) Scheme for the involvement of TSC-Rheb-TOR-S6K signaling in the stress response.

Reduced Rheb-TOR-S6K signaling confers resistance to oxidative stress

Since increasing systemic Rheb-TOR-S6K signaling sensitizes flies to oxidative stress, we tested whether reducing Rheb-TOR-S6K signaling in adult flies provides resistance to oxidative stress. We reduced Rheb-TOR-S6K signaling in adults by expressing the Tsc1/2 complex, Tsc2, or dominant-negative forms of TOR (FRB fragment) or S6K (KQ, kinase-dead form).

Co-overexpression of *Tsc1* and *Tsc2* during larval development can severely reduce cell growth by antagonizing TOR activity (Gao and Pan, 2001; Potter et al., 2001; Tapon et al., 2001). However, we were able to obtain viable adults co-overexpressing both *Tsc1* and *Tsc2* with *hs-GAL4*; co-overexpression of *Tsc1* and *Tsc2* with *da-* or *tub-GAL4* is early larval lethal (data not shown) similarly to *Rheb* mutants (Patel et al., 2003; Saucedo et al., 2003). We found that weak co-overexpression of *Tsc1* and *Tsc2* with *hs-GAL4* (*hs>Tsc1/2*) provides resistance to oxidative stress (Fig. 3B). Unlike *Tsc1/2* co-overexpression, overexpression of *Tsc1* or *Tsc2* alone does not suppress growth in larval tissues (presumably as a result of low GAP activity of Tsc2 without Tsc1 in vivo), thus we were able to overexpress *Tsc2* in adults using *da-GAL4* or *tub-GAL4*. Strong overexpression of *Tsc2* with *da-* or *tub-GAL4* (*da>Tsc2* and *tub>Tsc2*) flies provides resistance to oxidative stress similarly to *hs>Tsc1/2* flies (Fig. 3B). This is consistent with the observation that Tsc2 can exhibit GAP activity towards Rheb (Zhang et al., 2003). Similarly, we found that overexpression of a dominant-negative form of *TOR* (*TOR*^{FRB}) with *da-GAL4* (*da>TOR*^{FRB}) or a dominant-negative form of *S6K* (*S6K*^{KQ}) with *hs-* (*hs>S6K*^{KQ}) and *da-GAL4* (*da>S6K*^{KQ}) also conferred resistance to oxidative stress (Fig. 3B).

S6K is required for increased Rheb-TOR signaling to sensitize the whole organism to stress

To determine whether TOR and S6K play a role in the effects of *Rheb* overexpression on oxidative stress tolerance, we co-overexpressed *TOR*^{FRB} and *S6K*^{KQ} in flies overexpressing *Rheb* with *hs-GAL4* (*hs>Rheb*) and examined whether suppression of the sensitivity to 5% H_2O_2 can be observed. We found that overexpression of *TOR*^{FRB} can partially rescue the sensitivity of *hs>Rheb* flies to oxidative stress (5% H_2O_2) (Fig. 3C). TOR has been reported to have multiple downstream effectors; two well-characterized targets are 4E-BP and S6K. Co-overexpression of *S6K*^{KQ} in flies overexpressing *Rheb* fully rescued the stress response to 5% H_2O_2 (Fig. 3C) as well as to 10 mM paraquat (data not shown). These data suggest that increased TOR activity particularly through S6K activity can perturb the stress response (Fig. 3D).

Increased Rheb-TOR signaling in muscle sensitizes flies to oxidative stress

To determine whether particular tissues overexpressing *Rheb* contribute to the stress sensitivity observed in *hs>Rheb* flies, we used both constitutive and inducible GAL4 drivers to express *Rheb* in neurons, fat bodies and muscle tissue. We tested the effects of *Rheb* overexpression in neurons and fat bodies on the oxidative stress response,

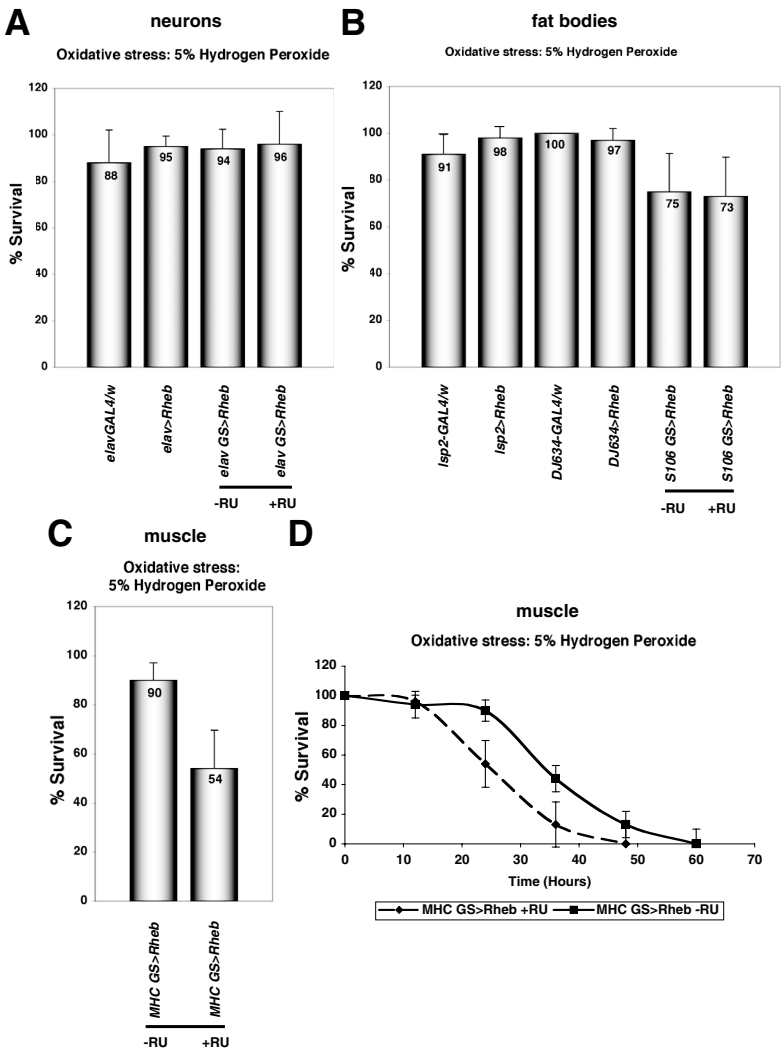

Fig. 4. Increased Rheb-TOR signaling in muscle but not in neurons or fat bodies sensitizes adult flies to oxidative stress. (A) Overexpression of *Rheb* in neurons using the pan-neural driver, *elav-GAL4* and the inducible (with RU486) GeneSwitch (GS) *elav GS-GAL4* does not sensitize flies to oxidative stress. (B) In addition, overexpression of *Rheb* in the fat bodies with *lsp2-GAL4* or *DJ634-GAL4* does not sensitize flies to oxidative stress. Similarly, using the GeneSwitch *S106 GS-GAL4*, which allows expression in the abdominal fat body does not sensitize flies to oxidative stress. All values reported in A-C represent the mean survival of the indicated genotype 24 hours after exposure to 5% H_2O_2. (C) By contrast, flies overexpressing *Rheb* in muscle tissue (fed RU486) using the pan-muscle Gene Switch (GS) driver, myosin heavy chain (*MHC*) *GS-GAL4* are sensitive to oxidative stress compared with control *MHC GS>Rheb* flies (not fed RU486). At 24 hours, the mean survival of MHC GS>Rheb flies not fed RU486 (n=102) is 90% whereas it is only 54% for MHC GS>Rheb flies fed RU486 (n=96, P=0.0084) (C). (D) A Kaplan-Meier survival plot further reveals the sensitivity of *MHC GS>Rheb* +RU flies to oxidative stress compared with *MHC GS>Rheb* –RU flies. Error bars represent the s.d. Statistical comparison (T-test): all P values are based on comparison of flies fed RU486 with those not fed RU486.

because expression of *JNK* (in neurons) and *foxo* (in fat bodies), can increase *Drosophila* adult lifespan and provide stress resistance (Giannakou et al., 2004; Hwangbo et al., 2004; Wang et al., 2003). We also included muscle in our analysis as a result of our observation of early senescence of locomotor activity in flies overexpressing *Rheb*.

To examine the stress response of flies overexpressing *Rheb* in adult neural tissue, we used the pan-neural *elav-GAL4* and *elav GS-GAL4* drivers. We found that constitutive expression of *Rheb* in all neurons did not affect development to adulthood nor did it sensitize adult flies to oxidative stress (Fig. 4A). For inducible expression, we used the GeneSwitch system that uses an inducible progesterone-receptor–GAL4 fusion. This allows expression from *UAS*-transgenes (Osterwalder et al., 2001; Roman et al., 2001) after feeding of the antiprogestin, RU486, and allowed us to bypass the embryonic lethality resulting from *Rheb* overexpression with many constitutive tissue-specific GAL4 drivers. We found that *elav GS>Rheb* flies (± RU) are also insensitive to oxidative stress (Fig. 4A); together these data suggest that expression of *Rheb* in neurons does not sensitize the whole organism to stress. To examine the stress response in flies overexpressing *Rheb* in the adult fat bodies, we used *lsp2-GAL4* as used by Teleman et al. (Teleman et al., 2005) to overexpress *Dp110* (the catalytic subunit of PI3K) in adult fat bodies. We found that *lsp2>Rheb* flies are insensitive to oxidative stress (Fig. 4B). We also used *DJ634-GAL4*, which allows expression primarily in the adult fat body and muscle (Kapahi et al., 2003). Adult flies expressing *Tsc2*, TOR^{FRB} or $S6K^{KQ}$ with *DJ634-GAL4* lived longer (Kapahi et al., 2004); however, we found that *DJ634>Rheb* flies were also insensitive to oxidative stress (Fig. 4B). To further examine the effects of overexpressing *Rheb* in adult fat bodies, we used the *S106 GS-GAL4* (Giannakou et al., 2004; Hwangbo et al., 2004) to express *Rheb* in the abdominal fat body but also found these flies to be insensitive to oxidative stress (Fig. 4B). These data together imply that expression of *Rheb* in the fat bodies probably does not sensitize the whole organism to stress. In contrast to neurons and fat bodies, expression of *Rheb* in muscle showed a significant increase in sensitivity to oxidative stress. Using the pan-muscle driver, *MHC* (myosin heavy chain) *GS-GAL4* (Wang et al., 2003); we found that *MHC GS>Rheb* flies (+RU) are sensitive to oxidative stress (compared with *MHC GS>Rheb*, –RU flies) (Fig. 4C,D). We also used *24B-GAL4* (Brand and Perrimon, 1993; Kapahi et al., 2004), which is expressed in muscle and fat body; however, we did not obtain many viable adults. These data together suggest that muscle might be one of the tissues affected in *hs>Rheb* flies when exposed to oxidative stress.

Increased Rheb-TOR-S6K activity also sensitizes flies to starvation

Because alteration of Rheb-TOR-S6K signaling can affect the stress response to oxidative stress, we tested whether Rheb-TOR-S6K signaling can influence the stress response to yet another stress, nutrient starvation. We found that flies overexpressing *Rheb* or $S6K^{STDETE}$ were also sensitive to nutrient starvation (fed PBS only) (Fig. 5A). By contrast, we found that flies overexpressing either *Tsc2* alone or $S6K^{KQ}$ were resistant to nutrient starvation (Fig. 5B). These data suggest that Rheb-TOR-S6K signaling might affect the response to various forms of stress and may not be limited to oxidative stress.

Increased Rheb-TOR signaling through S6K promotes early senescence of locomotor activity

Fly strains that exhibit an increased resistance to stress or increased lifespan often perform well in negative geotaxis assays (Gargano et al., 2005; Mockett et al., 2001). These assays test the ability of flies to travel against gravity, an innate escape behavior. In our assays, flies were tapped down to the bottom of their vials; we then scored the number of flies that travel 5 cm against gravity in 10 seconds. We found that flies overexpressing *Rheb* with *hs-GAL4* (*hs>Rheb*) perform as well as control flies in these assays 5 days after eclosion (Fig. 6). However, 30 days after eclosion, *hs>Rheb* flies perform poorly compared with control flies (Fig. 6). We also found that co-overexpression of $S6K^{KQ}$ can rescue this defect (Fig. 6), suggesting that increased TOR signaling through S6K is responsible for the observed early senescence of locomotor behavior.

Discussion

We have shown that altering levels of Rheb-TOR-S6K activity can influence the stress response. We found that increasing Rheb-TOR-S6K signaling by overexpression of *Rheb*, TOR or a constitutive active form of S6K can sensitize flies to oxidative stress. By contrast, decreasing Rheb-TOR-S6K signaling by

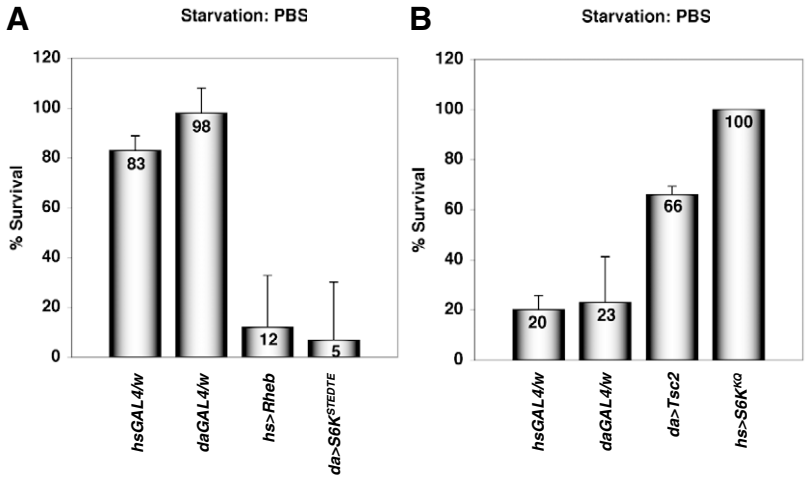

Fig. 5. Altering Rheb-TOR-S6K signaling can influence the response of adult flies to starvation stress. (A) The mean survival 24 hours after the start of PBS starvation is 83% for *hsGAL4/w* flies and 98% for *daGAL4/w* flies, but only 12% for hs>*Rheb* flies (n=100, P=0.0339) and 5% for *da>*$S6K^{STDETE}$ flies (n=100, P=0.0136). (B) The mean survival 36 hours after the start of PBS starvation is 66% for *da>Tsc2* flies (n=110, P=0.0059) and 100% for *hs>*$S6K^{KQ}$ flies (n=117, P=0.0019), but only 20% for *hsGAL4/w* flies (control) and 23% for *daGAL4/w* flies (control). Error bars represent the s.d. Statistical comparison (T-test): all P values are based on comparison of the overexpressor with controls (*hsGAL4/w* and *daGAL4/w*).

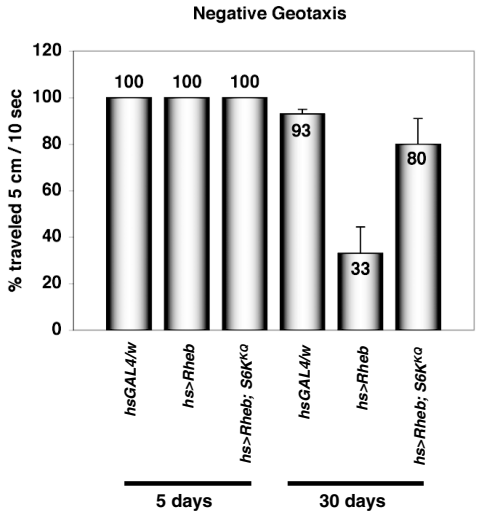

Fig. 6. Increased Rheb-TOR signaling through S6K promotes early senescence of negative geotaxis, the ability to travel against gravity. Although flies overexpressing *Rheb* (*hs>Rheb*) show no defect in this behavior 5 days after eclosion, *hs>Rheb* flies exhibit significant decrease in negative geotaxis 30 days post-eclosion (P=0.0002). Furthermore, co-overexpression of dominant-negative $S6K^{KQ}$ can rescue this defect in *hs>Rheb* flies. After 30 days, the mean percentage of flies traveling 5cm in 10 seconds is 93% for *hsGAL4/w* flies, 33% for *hs>Rheb* flies and 80% for *hs> Rheb;* $S6K^{KQ}$ flies (P=0.0705, NS). Error bars represent the s.d. Statistical comparison (T-test): P values are based on comparison of the overexpressor with the control (*hsGAL4/w*).

expression of the Rheb GAP Tsc2 or dominant-negative forms of TOR and S6K provides flies with resistance to oxidative stress. We also found that co-overexpression of dominant-negative S6K can rescue the sensitivity to oxidative stress observed in *Rheb*-overexpressing flies suggesting that S6K is an important effector of TOR that increases stress sensitivity. Furthermore, our data suggest that levels of Rheb-TOR signaling in muscle tissue might be important because overexpression of *Rheb* in muscle sensitizes flies to oxidative stress.

The importance of the Rheb-TOR signaling to the stress response is not limited to oxidative stress. We found that alteration of this pathway similarly affects the starvation stress response indicating that increased TOR signaling could also influence the response to other forms of stress. In addition, we found that increasing Rheb-TOR signaling in the whole organism results in early senescence of locomotor activity. Further, we found that co-overexpression of dominant-negative S6K can rescue the early senescence of locomotor activity in flies overexpressing *Rheb*, suggesting the importance of S6K as an effector of TOR in aging and age-related disease. A recent study in yeast also points to the importance of the TOR signaling in oxidative stress response and aging (Powers et al., 2006).

Several defense mechanisms exist to protect cells from oxidative stress such as anti-oxidant enzymes (Finkel and Holbrook, 2000). We did not find differences in the expression of anti-oxidant enzyme genes (Cu/Zn SOD, MnSOD, catalase or thioredoxin reductase-1) nor in anti-oxidant activities of catalase, Cu/Zn SOD or MnSOD in *Rheb*-overexpressing flies under normal conditions (data not shown). We also did not observe a difference in the gene expression of anti-oxidant enzymes (catalase and MnSOD) nor in catalase activity in flies overexpressing *Rheb* after exposure to 5% H_2O_2. Upon oxidative or starvation stress, a key transcription factor in metazoans, FOXO, promotes the expression of several stress-defense genes, including the gene encoding *Drosophila 4E-BP* (*Thor*) and those encoding anti-oxidant enzymes and chaperones (Essers et al., 2004; Greer and Brunet, 2005; Murphy et al., 2003; Wang et al., 2005). We found strong *4E-BP* expression after exposure to oxidative stress in flies overexpressing *Rheb* (data not shown), suggesting that Rheb-TOR signaling does not sensitize the whole organism to stress by antagonizing FOXO activity.

Several recent studies suggest a need for deceleration of protein translation during stress (Holcik and Sonenberg, 2005; Tettweiler et al., 2005). Increased Rheb-TOR-S6K signaling may stimulate 5′-cap-dependent translation resulting in increased sensitivity of the organism to stress. Increased Rheb-TOR signaling could stimulate 5′-cap-dependent translation by (1) mediating assembly of the pre-translation initiation complex by increasing S6K activity (Holz et al., 2005) or by (2) enabling 5′-cap dependent translation by inhibiting 4E-BP. However, flies mutant for *4E-BP* or *S6K* display only mild growth defects during development (Miron et al., 2001; Montagne et al., 1999). Although their importance in translation control in adults needs to be investigated further, increasing Rheb-TOR signaling may not sensitize the whole organism merely by promoting 5′ cap dependent translation through inhibition of 4E-BP or by stimulation of S6K. Furthermore, these data allow for the possibility that TOR or S6K can influence the stress response through unknown protein and gene targets unrelated to protein translation such as relieving the cytoplasmic retention by TOR of important stress response transcription factors (Beck and Hall, 1999; Powers et al., 2006).

The failure of flies with increased Rheb-TOR-S6K signaling to elicit a stress response could result in the accumulation of oxidative damage resulting in premature aging and degeneration. One sign of early aging that we observe in flies overexpressing *Rheb* is the early senescence of locomotor activity (in particular, negative geotaxis). The senescence of negative geotaxis is observed in older flies as well as in short-lived strains that exhibit sensitivity to oxidative stress (Gargano et al., 2005; Mockett et al., 2001). Thus, the early senescence of locomotor activity in *Rheb*-overexpressing flies suggests the possibility for early aging and degeneration of these flies. The overexpression of either dominant-negative TOR or S6K can rescue the effects of *Rheb* overexpression on negative geotaxis implying that increased TOR signaling through S6K may be responsible for the early senescence or degeneration of locomotor activity.

Decreasing TOR activity results in increased lifespan in several organisms (Kapahi et al., 2004; Powers, 3rd et al., 2006; Vellai et al., 2003) and has recently been shown to play an important role in age-related neurodegenerative disease (Inoki et al., 2005a; Nelson et al., 2005; Ravikumar et al., 2004). Although we cannot exclude the possibility that the early senescence of locomotor activity in *Rheb*-overexpressing flies is due to long-term neural degeneration in these flies, it is possible that muscle degeneration could explain this behavior, because the overexpression of *Rheb* in muscle can sensitize flies to oxidative stress. Increased apoptosis is observed in leg and thoracic (which includes flight muscle) muscles in aging

flies (Zheng et al., 2005) providing an attractive mechanism to explain the decreased locomotor activity of aged flies. Increased Rheb-TOR signaling in skeletal muscle may accelerate the process by inhibiting the stress response. Alternatively, levels of Rheb-TOR signaling in myocardial tissue could be an important determinant of lifespan because decreasing insulin signaling in *Drosophila* adults can prevent the decline of cardiac performance with age (Wessells et al., 2004). Further work using *Drosophila* may provide valuable insights into efforts to control TOR and S6K activity to treat several age-related diseases and/or their progression.

Materials and Methods

Fly strains and maintenance

Wild-type and dominant-active or -negative forms of Rheb-TOR-S6K pathway components were ubiquitously overexpressed by combining *arm-*, *hs-*, *da-*, *act-* or *tub*-GAL4 drivers with *UAS-Tsc1, UAS-Tsc2* (Tapon et al., 2001), *UAS-Tsc2* (Tapon et al., 2001), *UAS-Rheb* (Patel et al., 2003), *UAS-TOR* (Hennig and Neufeld, 2002), *UAS-TOR^{TED}* (Hennig and Neufeld, 2002), *UAS-TOR^{FRB}* (Hennig and Neufeld, 2002), *UAS-S6K^{STEDTE}* (Barcelo and Stewart, 2002) and *UAS-S6K^{KQ}* (Barcelo and Stewart, 2002). *Rheb* was overexpressed in adult neurons using *elav-GAL4* and *elav GS-GAL4* (Osterwalder et al., 2001; Roman et al., 2001; Wang et al., 2003), in fat bodies using *lsp2-GAL4* (Teleman et al., 2005), *DJ634-GAL4* (Kapahi et al., 2004) and *S106 GS-GAL4* (Giannakou et al., 2004; Hwangbo et al., 2004; Roman et al., 2001) and in muscle using *24B-GAL4* (Brand and Perrimon, 1993; Kapahi et al., 2004) and *MHC GS-GAL4* (Osterwalder et al., 2001; Wang et al., 2003).

Northern analysis

Total RNA was isolated using TRIzol reagent (Invitrogen). 5 μg of *Drosophila* adult total RNA was separated on a denaturing 1% agarose-1.5% formaldehyde gel, transferred to nylon membrane and probed with [α-^{32}P]dATP-labeled probes for *Rheb* and *actin5C* genes.

Western analysis

80 μg protein were separated on an 8% SDS-polyacrylamide gel. Phosphorylated S6K was detected using anti-phospho-S6K T398 antibody from Cell Signaling Technology.

Weight analysis

The mean weight of individual males and females of each genotype was determined with a Mettler ME30 precision scale (0.001-10 mg). Flies were grown and maintained at low density and weighed (n=50) 5 days post eclosion. Weight measurements were repeated three times.

Developmental rate

Embryos for each cohort were collected for 2 hours at 25°C. 25 embryos were immediately transferred to each food vial and allowed to develop at low density. 200 embryos were used for each genotype. Emerging adults were scored every 12 hours.

Stress tests

Embryos of each genotype were collected for 2 hours and then separated into new food vials (25 embryos per vial) to allow development to progress at low density. Newly eclosed adults were removed from their vials, maintained at low density (ten flies per vial) and allowed to mate for 5 days before exposure to each stress. Each stress test consisted of three to five vials, ten adult males per vial, repeated in three different experiments. Two different protocols were used to test sensitivity to oxidative stress. In one protocol (Fig. 3A,B), each genotype was placed into a vial containing a 3 mm Whatman filter wetted with a solution of 5% H_2O_2 in 5% sucrose/PBS. Vials were scored every 12 hours for the number of dead flies. Because *Rheb*-overexpressing adults were found to be sensitive to a continuous diet of 5% sucrose/PBS, a second type of oxidative stress test was performed by placing adults daily for 6 hours into a vial containing a 3 mm Whatmann filter wetted with a solution of 5% H_2O_2 or 20 mM paraquat (methyl viologen) in 5% sucrose/PBS (Fig. 2A,B and Fig. 3C). Adults were then transferred to normal growth medium. This treatment was repeated daily until the end of the experiment. Vials were scored every 24 hours for the number of dead flies. For starvation stress tests, flies were placed in a vial containing a 3 mm Whatmann filter wetted with PBS alone (Fig. 5A,B). Vials were scored every 12 hours for the number of dead flies. All stress tests were performed at 25°C.

Conditional expression of Rheb

Conditional expression of *Rheb* was effected by using the GeneSwitch (GS) system (Osterwalder et al., 2001; Roman et al., 2001; Wang et al., 2003). Tissue-specific conditional expression was obtained using *elav GS-GAL4* (pan-neural), *S106 GS-GAL4* (abdominal fat body) and *MHC GS-GAL4* (pan-muscle). GS-GAL4 activity was induced by feeding flies 400 μg/ml RU486 for 24 hours; flies were then transferred to new vials for stress tests performed as described above.

Negative geotaxis assay

Ten adult males per empty vial were tapped down to the bottom of the vial and the number of flies that could travel 5 cm towards the top of each vial in 10 seconds was scored. Approximately 30-50 flies were used for each cohort; each experiment was repeated three times.

We would like to thank Frank Laski (UCLA) for his helpful advice on *Drosophila* experiments and critical comments on the manuscript. This work was supported by grants from the National Institutes of Health to P.H.P. (Ruth L. Kirschstein National Research Service Award (GM07185)) and to F.T. (CA41996). The authors would also like to thank the Bloomington Stock Center for fly stocks as well as Naoto Ito (Harvard/ MGH) and Iswar Hariharan (UC Berkeley) for *UAS-Tsc1*, *UAS-Tsc2* and *UAS-Tsc2* stocks, Thomas Neufeld (University of Minnesota) for *UAS- TOR^{FRB}* stock and Heinrich Jasper (U. Rochester) for the *elav-GAL4* and *GeneSwitch- GAL4* stocks.

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