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MAP4K3 regulates body size and metabolism in Drosophila

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ABSTRACT

The TOR pathway mediates nutrient-responsive regulation of cell growth and metabolism in animals. TOR Complex 1 activity depends, amongst other things, on amino acid availability. MAP4K3 was recently implicated in amino-acid signaling in cell culture. We report here the physiological characterization of *MAP4K3* mutant flies. Flies lacking *MAP4K3* have reduced TORC1 activity detected by phosphorylation of S6K and 4EBP. Furthermore *MAP4K3* mutants display phenotypes characteristic of low TORC1 activity and low nutrient availability, such as reduced growth rate, small body size, and low lipid reserves. The differences between control and *MAP4K3* mutant animals diminish when animals are reared in low-nutrient conditions, suggesting that the ability of TOR to sense amino acids is most important when nutrients are abundant. Lastly, we show physical interaction between MAP4K3 and the Rag GTPases raising the possibility they might be acting in one signaling pathway.

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Introduction

The multiprotein complex TORC1, containing TOR kinase, is a central regulator of cellular growth and metabolism in animals (Bjornsti and Houghton, 2004; Guertin and Sabatini, 2007). It is activated by a number of inputs relating to cellular energy and nutrient status. These include insulin, glucose, cellular energy levels and amino acid availability (Avruch et al., 2006; Hay and Sonenberg, 2004). In response, TORC1 activates protein synthesis via a number of mechanisms including activation of the ribosomal S6 kinase (S6K), repression of the translational inhibitor 4E-BP, and promotion of ribosome biogenesis via myc (Hay and Sonenberg, 2004; Teleman et al., 2008). In particular since TORC1 is a master regulator of protein biosynthesis, its regulation by amino acids, the building blocks of proteins, likely constitutes an important regulatory feedback mechanism. Furthermore, the importance of amino acid signaling to TOR is highlighted by the observation that circulating amino acids are elevated in humans with obesity, where they have been shown to activate TORC1 activity and modulate glucose metabolism (Krebs, 2005). Despite this, our understanding of the molecular mechanism by which amino acids regulate TOR remains fragmentary.

Three protein complexes have recently been implicated in the activation of TORC1 in response to amino acids. The human class III PI3K (phosphoinositide 3-kinase) hVps34 is activated by amino acids via a calcium dependent mechanism (Gulati et al., 2008). This leads to accumulation of phosphatidylinositol 3-phosphate (PI(3)P) in cells, which is thought to cause the recruitment of proteins recognizing PI(3)P to early endosomes, forming an intracellular signaling platform that leads to TORC1 activation (Byfield et al., 2005; Nobukuni et al., 2005). This feature of the pathway may be specific for vertebrates, as flies mutant for Vps34 have been reported to not have TORC1 signaling defects (Juhasz et al., 2008). Recently, two groups discovered that Rag GTPases mediate amino acid signaling to TORC1 (Kim et al., 2008; Sancak et al., 2008). The emerging picture is that amino acids change the GDP/GTP loading of the Rag GTPases, thereby stimulating the binding of Rag heterodimeric complexes to TORC1. This in turn causes TORC1 to change its intracellular localization, perhaps relocalizing it to vesicles containing the activator Rheb. This mechanism appears to be evolutionarily conserved from flies to humans.

The third protein recently identified as a mediator of amino acid signaling to TOR is MAP4K3 (Findlay et al., 2007). Findlay et al., (2007) showed in HeLa cells that the kinase activity of MAP4K3 is activated in the presence of amino acids. In turn, MAP4K3 is required for TOR to phosphorylate its targets S6K and 4E-BP1 in response to amino acid sufficiency. The cell-culture data also suggest this mechanism is conserved from flies to humans as knockdown of Drosophila MAP4K3 causes a reduction in TOR activity.

Although considerable progress has been made, important questions remain unanswered. The role of TORC1 activity in vivo has been well studied in flies and mice, but fundamental issues regarding the regulation of TOR by amino acids have thus far only been explored in vitro in cell culture. To assess the functional significance of the ability of TORC1 to sense amino acids in the organismal context we have made

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use of a Drosophila mutant for MAP4K3 (CG7097). We show that dMAP4K3 mutant flies have reduced TOR activity, detected by phosphorylation of TOR targets. dMAP4K3 mutants are viable but display physiological aberrations emblematic of animals starved of nutrients: *MAP4K3* mutant flies have retarded growth, reduced size, and low lipid reserves. Both the biochemical results and the phenotypes indicate that MAP4K3 modulates, but is not absolutely required, for TOR activity *in vivo*. This is similar to what is observed with other modulators of the pathway, such as Melted. Unexpectedly, the function of MAP4K3 is most required when nutrient conditions are rich.

Materials and methods

Expression constructs and fly lines

Flies containing the l(2)SH1261 P-element insertion were obtained from the Bloomington Stock Center. The construct for expressing HA-tagged MAP4K3 in S2 cells was built by subcloning cDNA GH26057 (BACPAC Resources Center) as a *Eco*RV–*Sal*I fragment into pRmHa3b, containing a copper-inducible promoter. An HA-tag was introduced at the C-terminus of MAP4K3 into the *Nhe*I site, using oligos 5'-CTAGCTACCCCTACGACGTCCCGGACTATGCCTAA-3' and 5'-CTAGTTAGG-CATAGTCCGGGACGTCGTAGGGGTAG-3'. Similar constructs were built for RagA and RagC by amplifying their coding sequences from cDNA using oligos 5'GCGGCCGCAAGAAAAAGGTGTTACTGATGGG-3' and 5'-CTCGAGCGGCAAATGGAGTTATGGAA-3' for RagA and 5'-GCGG-CCGCAGCTACGATGATGATGACTATCC-3' and 5'-CTCGAGTTTTT-TACGCTGCTCTGTGA-3' for RagC. A FLAG-tag was introduced into the *Not*I site at the N-terminus using oligos 5'-GGCCATGGACTACAAGGAC-GACGACGACAAG-3' and 5'-GGCCCTTGTCGTCGTCGTCCTTGTAGTCCAT-3'. All final constructs were verified by sequencing. Mutant versions of MAP4K3, RagA and RagC were generated by standard point mutagenesis. MAP4K3 mRNA levels were tested by quantitative RT-PCR using oligos 5'-AACGTGGACAGCATTGTTTG-3' and 5'-CTCTCCAAGGCC-ACAACC-3'.

In vivo growth analyses and food conditions

Flies were grown under “growth controlled” conditions as described previously (Teleman et al., 2005a). Flies were grown on a rich food diet consisting of 3 L water, 36 g agar, 54 g dry yeast, 30 g soya powder, 66 g sirup, 240 g malt extract, 240 g corn powder, 18.6 mL propionic acid and 7.2 g nipagin. For low-nutrient challenge, 20% food was prepared by mixing with PBS/0.6% agarose. Triglyceride measurements and size measurements were performed as described (Teleman et al., 2005a).

Immunoprecipitation and immunoblotting

Immunoprecipitations and immunoblotting were performed as described previously (Teleman et al., 2005b). Anti-HA (Roche, 11867423001); anti-FLAG (Sigma, F1804) anti-pS6K(Thr398) (Cell Signaling 9209 S); anti-pT37/46 4EPB1 (Cell Signaling 9459 S); anti-engrailed (Developmental Studies Hybridoma Bank).

Results

Drosophila MAP4K3 mutants are viable

In order to study the *in vivo* function of amino acid sensing by MAP4K3, we screened a panel of publicly available Drosophila lines harboring P-element insertions in or near the dMAP4K3 (CG7097) locus for loss of dMAP4K3 expression. The l(2)SH1261 P-element is inserted in the second intron of dMAP4K3 (Fig. 1A). By quantitative RT-PCR, we found that flies homozygous for the l(2)SH1261 insertion have expression levels of dMAP4K3 that are only 1% that of control flies (Fig. 1B). We back-crossed female flies harboring the l(2)SH1261 insertion to w^{1118} flies for four generations in order to obtain two fly stocks with similar genetic backgrounds, differing by presence or absence of the l(2)SH1261 insertion. The resulting stocks were then used for all experiments described here, and we will refer to such flies homozygous for the l(2)SH1261 insertion as *MAP4K3* mutant flies, and the w^{1118} stock as control.

MAP4K3 mutant flies were able to reach adulthood, looked normally patterned, and were fertile. Nonetheless, they were not completely normal as they had reduced viability compared to control flies. Only 77% of *MAP4K3* mutant first-instar larvae reached adulthood, compared to 91% of controls (*t*-test 0.001, Fig. 1C). Within the first 2 days of life, 18% of *MAP4K3* mutants died, compared to 7% of controls (*t*-test <0.001, Fig. 1C), suggesting *MAP4K3* mutants are generally weaker than controls.

MAP4K3 mutants have reduced TOR activity levels in vivo

Knockdown of MAP4K3 in Drosophila S2 cells has been reported to reduce TOR activity (Findlay et al., 2007). We asked whether MAP4K3 mutant animals also have lower TOR activity compared to controls. We extracted protein from control and MAP4K3 mutant adult males and assayed TOR activity by detecting phosphorylation of two TOR targets, S6K and 4EBP (Fig. 2). MAP4K3 mutants compared to control animals had significantly reduced S6K and 4EBP phosphorylation levels (Fig. 2). As an additional control, we generated animals bearing a precise excision of the l(2)SH1261 P-element insertion, which should revert the lesion causing the *MAP4K3* mutation, and hence the loss-of-function phenotype. In these animals, phosphorylation of S6K and 4EBP were returned to normal levels (Fig. 2).

MAP4K3 mutants have retarded growth and reduced size

We asked whether *MAP4K3* mutant flies display phenotypes canonical of reduced TOR activity, such as reduced organismal growth rate and smaller final body size in part due to smaller cells. When equally staged *MAP4K3* mutant and control first-instar larvae were seeded at defined density in vials containing normal laboratory food, which is rich in amino acids and other nutrients (hereafter referred to as “controlled growth conditions”), *MAP4K3* mutants were delayed in pupation relative to controls by almost 2 days (Fig. 3A). We could not detect any defects in ecdysone signaling in *MAP4K3* mutants, assayed by quantitative RT-PCR of transcriptional targets of the pathways (Supplementary Fig. 1). Instead, *MAP4K3* mutants displayed a slower growth rate during larval stages. *MAP4K3* mutant larvae were significantly smaller than equally aged control larvae, as was evident by visual inspection 4 and 5 days after egg laying (Fig. 3B) and when quantified (Fig. 3C). Growth curves obtained by weighing larvae at successive days of development showed that *MAP4K3* mutants accumulated mass more slowly than controls (Fig. 3D). *MAP4K3* mutants also displayed a larger inter-individual variation in growth rate compared to controls, as can be seen by the larger standard deviation in the size (Fig. 3C) and the shallower pupation curve of the *MAP4K3* mutant population compared to controls (Fig. 3A). *MAP4K3* mutant adult flies were also significantly reduced in size, as could be quantified by measuring wing area. Mutant wings were roughly 20% smaller than wings from control flies (*t*-test $=1\times10^{-7}$, Fig. 3E). The difference in size between mutant and control adults was smaller than the difference between co-aged larvae due to the 2-day extended larval growth period of the mutants allowing them to grow to near normal size before pupation. In order to quantify cell size in *MAP4K3* mutant adults, we measured hair number per unit area in *MAP4K3* mutant and control wings (Fig. 3F). *MAP4K3* mutants had a significantly reduced cell size compared to controls (*t*-test $=2\times10^{-5}$, Fig. 3F). Since only strong growth defects can be readily observed using mitotic clones, we could not detect striking

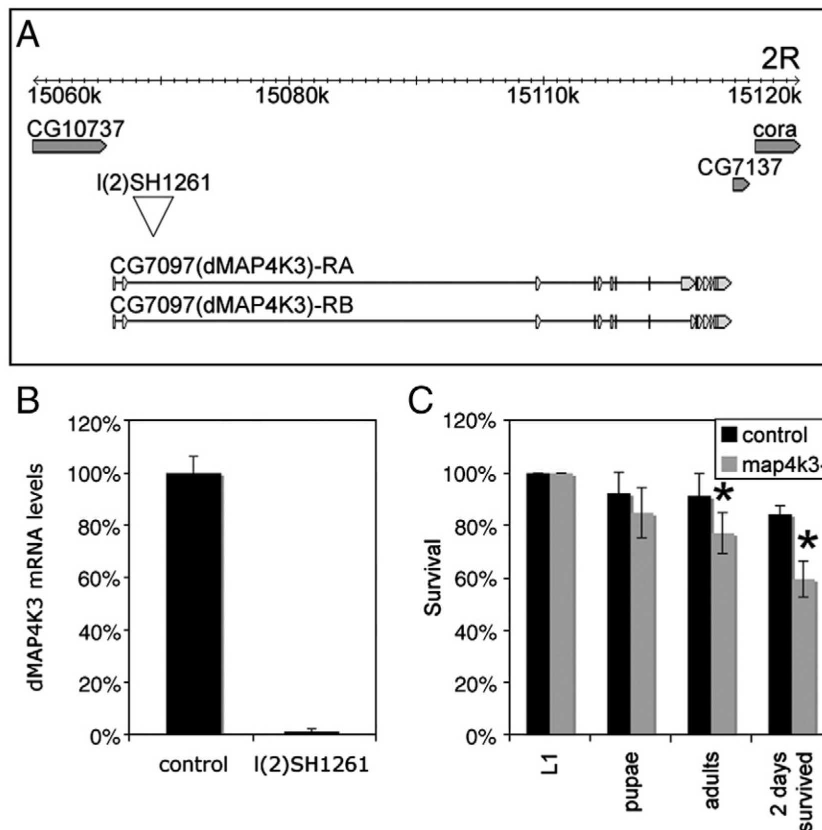


Fig. 1. MAP4K3 mutant flies are viable. (A) Overview of the dMAP4K3 (CG7097) genomic locus, showing insertion point of the l(2)SH1261 P-element relative to the two dMAP4K3 splice isoforms. l(2)SH1261 is inserted in the second intron of the gene. (B) Quantitative RT-PCR on dMAP4K3 mRNA normalized to rp49. Flies homozygous for the l(2)SH1261 insertion have 1% the level of MAP4K3 expression as compared to w^{1118} flies ("control"). Error bars indicate standard deviation. (C) Survival curve for control (dark bars) and MAP4K3 mutant flies (light bars). The majority of MAP4K3 mutant flies survive to adulthood ("adults") although viability is slightly but significantly reduced compared to control flies (t-test = 0.001). A significant number of MAP4K3 mutant adults die the first 2 days of life (circa 20%), significantly more than controls (t-test <0.001). Error bars indicate standard deviation and * indicates significance.

defects in MAP4K3 mutant clones in the wing disc (Supplementary Fig. 2).

MAP4K3 mutants are lean

Signaling through the insulin and TOR pathways has a significant impact on fly metabolism (Baker and Thummel, 2007; Teleman et al., 2005b). We asked whether *MAP4K3* mutant animals have altered metabolic characteristics compared to controls. Triglyceride levels are one important metabolic parameter in Drosophila, as flies store most of their energy in the form of triglycerides in their adipose tissue (Van der Horst, 2003). We reared mutant and control animals under controlled growth conditions, and measured total body triglycerides, normalized to total body protein to control for animal size. *MAP4K3* mutant animals had roughly 40% less fat than control animals (t-test < 0.01, Fig. 4A). This phenotype was reversed in animals bearing a precise excision of the l(2) SH1261 P-element insertion (t-test < 0.01, Fig. 4A). As a further control, flies trans-heterozygous for the l(2)SH1261 P-element insertion over a deficiency uncovering the MAP4K3 locus were also lean (Fig. 4B, t-test <0.01). The leanness of *MAP4K3* mutants could be reversed by expressing MAP4K3 via a UAS transgene together with the low-level ubiquitous driver daughterless-GAL4 (Fig. 4C, t-test < 0.01).

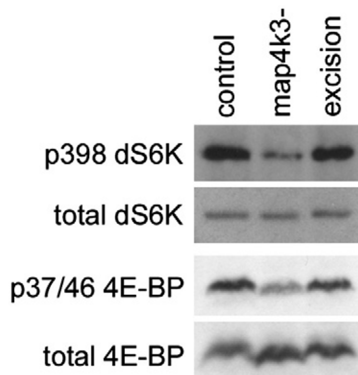


Fig. 2. MAP4K3 mutant flies have reduced TOR activity. Protein extracts from 5-day-old adult males of control, MAP4K3 mutant or l(2)SH1261 excision flies, probed for phospho- and total S6K and 4EBP protein as indicated.

The combination of growth and metabolic phenotypes displayed by *MAP4K3* mutants–reduced growth rate and low lipid levels–suggest that the mutant animals are reacting physiologically as if they were nutritionally starved, even though nutrients are available.

We postulated that the starvation-like phenotypes of *MAP4K3* mutant flies might be exacerbated by rearing animals in a condition where nutrient supply is limiting, rather than in high abundance as under standard laboratory conditions. To this end, we seeded first-instar larvae on food that had been diluted 1:5 with PBS/agarose, reducing the concentration of all nutrients. Under these conditions, both mutant and control animals took longer to pupate than under normal food conditions (Fig. 3A and A'). To our surprise, however, *MAP4K3* mutants were no longer disadvantaged in terms of growth rate, and pupated at the same time as control flies (Fig. 3A'). This finding is consistent with cell culture data showing that MAP4K3 is required for full activation of TOR in the presence of amino acids, but is less important for TOR activation in the absence of amino acids

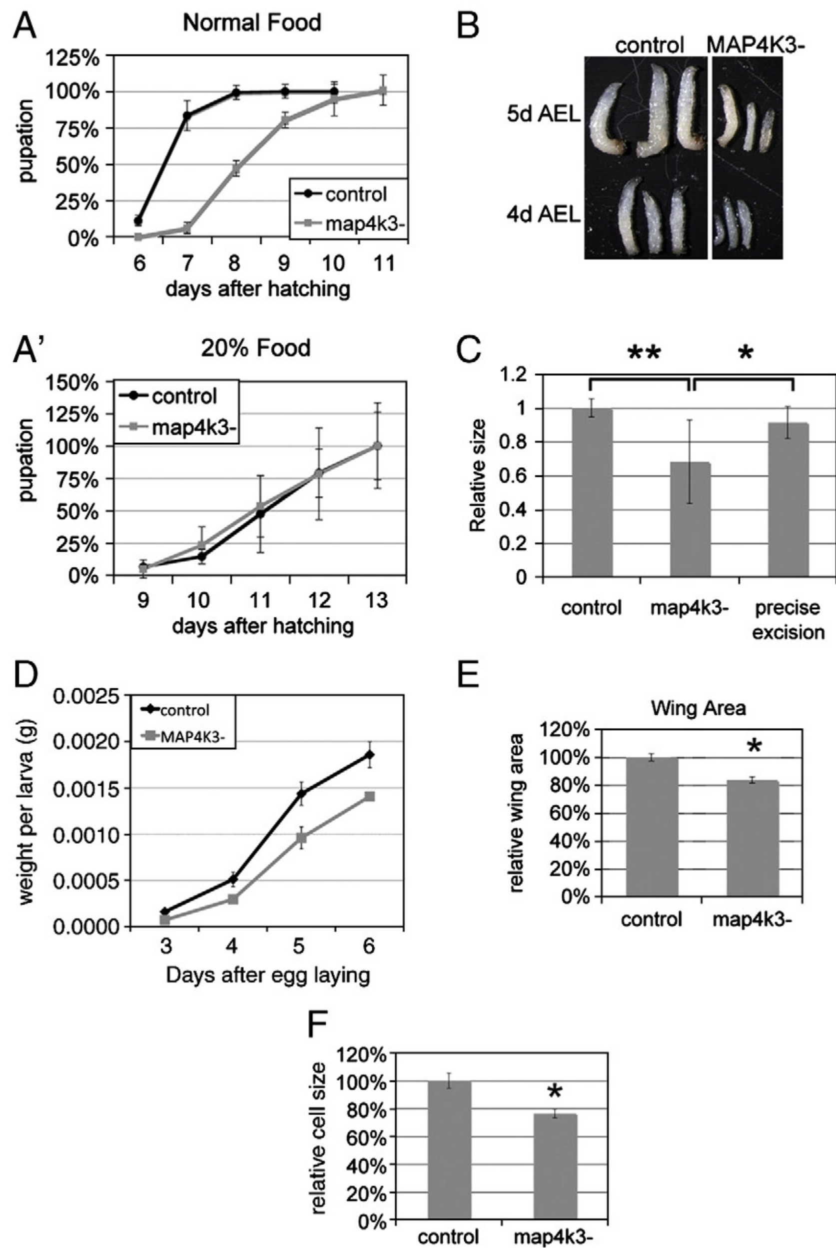


Fig. 3. MAP4K3 mutant flies display TOR-like growth phenotypes. (A,A') Pupation curves for control (dark graphs) and MAP4K3 mutant flies (light graphs). Flies were grown on (A) normal laboratory food or (A') normal laboratory food diluted 1:5 with PBS/agarose to generate a condition of limiting food supply. Error bars indicate standard deviation. (B) MAP4K3 mutant larvae are smaller than equally aged control larvae, both 4 and 5 days after egg laying (4d AEL and 5d AEL respectively). (C) Quantification of larval size of equally aged, male animals grown under controlled conditions (50 animals per vial). Size was quantified by measuring the area of individual larvae on pictures. Control, MAP4K3 mutant and l(2)SH1261 precise excision animals ("precise excision") as indicated. Error bars indicate standard deviation. ** and * indicate statistically significant differences (t-test = 0.002 and 0.01 respectively). (D) Growth curves of control and MAP4K3 mutant larvae seeded on normal laboratory food at a density of 50 larvae per vial in triplicate. Larval weights were measured on indicated days after egg laying. Error bars indicate standard deviation. (E) Relative wing size of control and MAP4K3 mutant flies. Error bars indicate standard deviation. * MAP4K3 mutant wings were significantly smaller (t-test $= 1 \times 10^{-7}$). (F) Wing cell size is reduced in MAP4K3 mutant animals compared to controls. Cell size in the wing was measured by counting hairs for an area of defined size, posterior to vein 5. Error bars indicate standard deviation. Mutant animals have significantly smaller cells than control animals (t-test $= 2 \times 10^{-5}$ indicated by *). In all cases (A–F), animals were grown under controlled conditions, seeded as first instars at a density of 50 animals per vial.

(Findlay et al., 2007). To test whether the difference in growth rate between control and *MAP4K3* mutant flies is specifically due to the sensing of amino acids, we generated food in which the two amino-acid rich ingredients, yeast extract and soy meal, were reduced to 20% their normal concentration whereas all other ingredients, including the carbohydrate-rich malt extract, were left normal. Whereas *MAP4K3* mutant animals displayed a growth delay relative to controls on normal laboratory food (Fig. 5A), both control and *MAP4K3* mutant animals grew equally slowly on low-amino acid food, so that the difference between the two genotypes was no longer statistically significant (Fig. 5B). We also assessed activation of TOR in control and *MAP4K3* mutant larvae grown on normal and low-amino acid food, by quantifying phosphorylation of S6K (Fig. 5C). On normal food, *MAP4K3* mutant animals had reduced levels of phospho-S6K compared to control animals (Fig. 5C, lanes 1 and 2). Whereas phosphorylation of S6K dropped in wildtype animals growing on low-amino acid food compared to normal food (Fig. 5C, lanes 1 and 3), this drop was not visible in MAP4K3 mutant animals (Fig. 5C, lanes 2 and 4). Together, these data suggest that MAP4K3 is required in vivo to activate TOR and spur growth when amino acid conditions are rich, whereas its activity is not as necessary when amino acid levels are limiting.

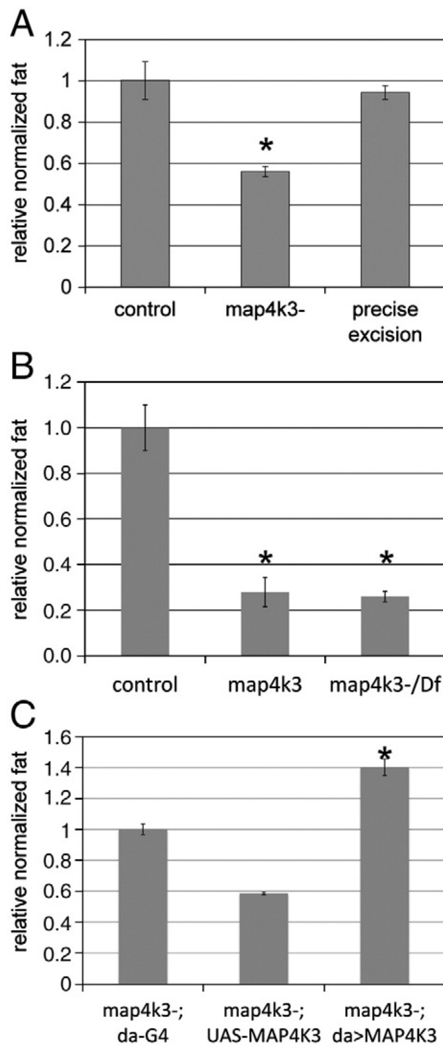


Fig. 4. MAP4K3 mutant flies are lean. (A) Relative triglyceride levels, normalized to total body protein, for control flies, MAP4K3- mutant flies homozygous for the l(2)SH1261 insertion ("map4k3-") and flies in which the P-element was precisely excised ("precise excision"). (B) Relative triglyceride levels, normalized to total body protein, for control flies, MAP4K3- mutant flies homozygous for the l(2)SH1261 insertion ("map4k3-"), and flies trans-heterozygous for the l(2)SH1261 insertion over the deficiency Df(2R) Exel6069 uncovering the MAP4K3 locus (map4k3-/Df). (C) Relative triglyceride levels, normalized to total body protein, for map4k3 mutant flies carrying either the daughterless-GAL4 driver ("map4k3-; da-G4"), or a UAS-MAP4K3 transgene ("map4k3-; UAS-MAP4K3") or both, resulting in ubiquitous low-level expression of MAP4K3 in the map4k3 mutant background ("map4k3-; da>MAP4K3"). In all cases, error bars indicate standard deviation and * indicates statistical significance (t-test <0.01).

MAP4K3 interacts physically with RagA and RagC

While working with MAP4K3, we noticed MAP4K3 protein contains a CNH domain at its C-terminal end. This domain is present in other kinases such as Citron kinase and Myotonic dystrophy kinase-related Cdc42-binding kinase (MRCKa), which interact with small GTPases such as Rho and Cdc42 (Madaule et al., 1995; Zhao and Manser, 2005). Therefore, we postulated that MAP4K3 might interact with a small GTPase in the TOR signaling pathway. To this end, we tested whether MAP4K3 can bind Rheb, an activator of TOR. We expressed HA-tagged MAP4K3 and myc-tagged Rheb in S2 cells, and performed immunoprecipitations in both directions (anti-HA and anti-myc), but were not able to detect any binding between MAP4K3 and Rheb (data not shown). Recently, a new set of GTPases have been reported to regulate TOR—the Rag GTPases (Kim et al., 2008; Sancak et al., 2008). Unlike Rheb, the Rag GTPases are important for TOR to be responsive to amino acids. To test whether MAP4K3 can bind Rag GTPases, we expressed HA-MAP4K3 together with FLAG-dRagA and FLAG-dRagC in S2 cells and immunoprecipitated RagA and RagC with anti-FLAG antibody (Fig. 6A). HA-MAP4K3 could be strongly detected in the FLAG immunoprecipitate (Fig. 6A, lane 3). As a control, HA-MAP4K3 could not be detected in the immunoprecipitate from lysates of cells not co-transfected with FLAG-RagA and FLAG-RagC (Fig. 6A lane 1) showing that immunoprecipitation of MAP4K3 required presence of the Rag proteins. As a further control for specificity, an unrelated HA-tagged protein (HA-Medea) was not co-immunoprecipitated despite being present in the lysate at equal levels as HA-MAP4K3 (Fig. 6A).

We then tested whether the interaction between MAP4K3 and the RagA/C complex is dependent on the presence of amino acids or the state of activation of TORC1. Inactivation of TORC1 with rapamycin had no detectable effect on binding between MAP4K3 and the RagA/C complex (Fig. 6B, lane 3), whereas amino acid removal caused a slight but reproducible reduction in binding (Fig. 6B, lane 2). As a control for effectiveness of the treatments, both rapamycin treatment and amino acid removal caused complete dephosphorylation of the TOR substrate S6K (Fig. 6B).

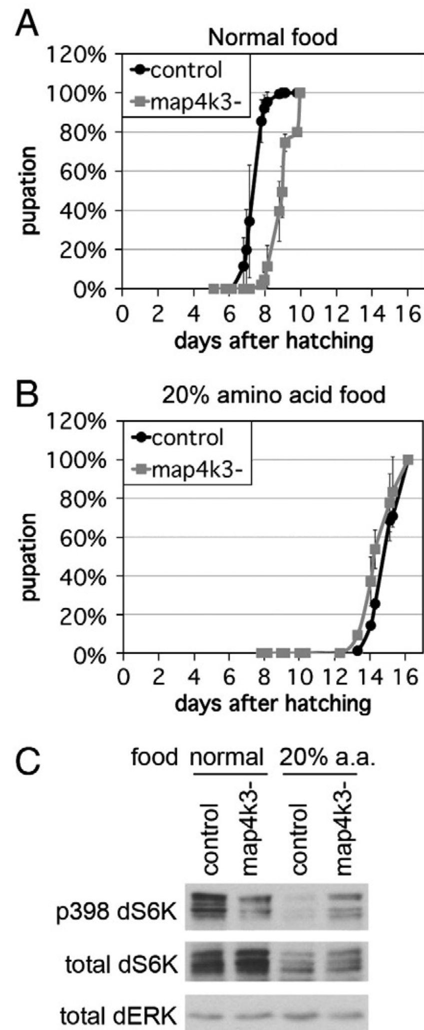
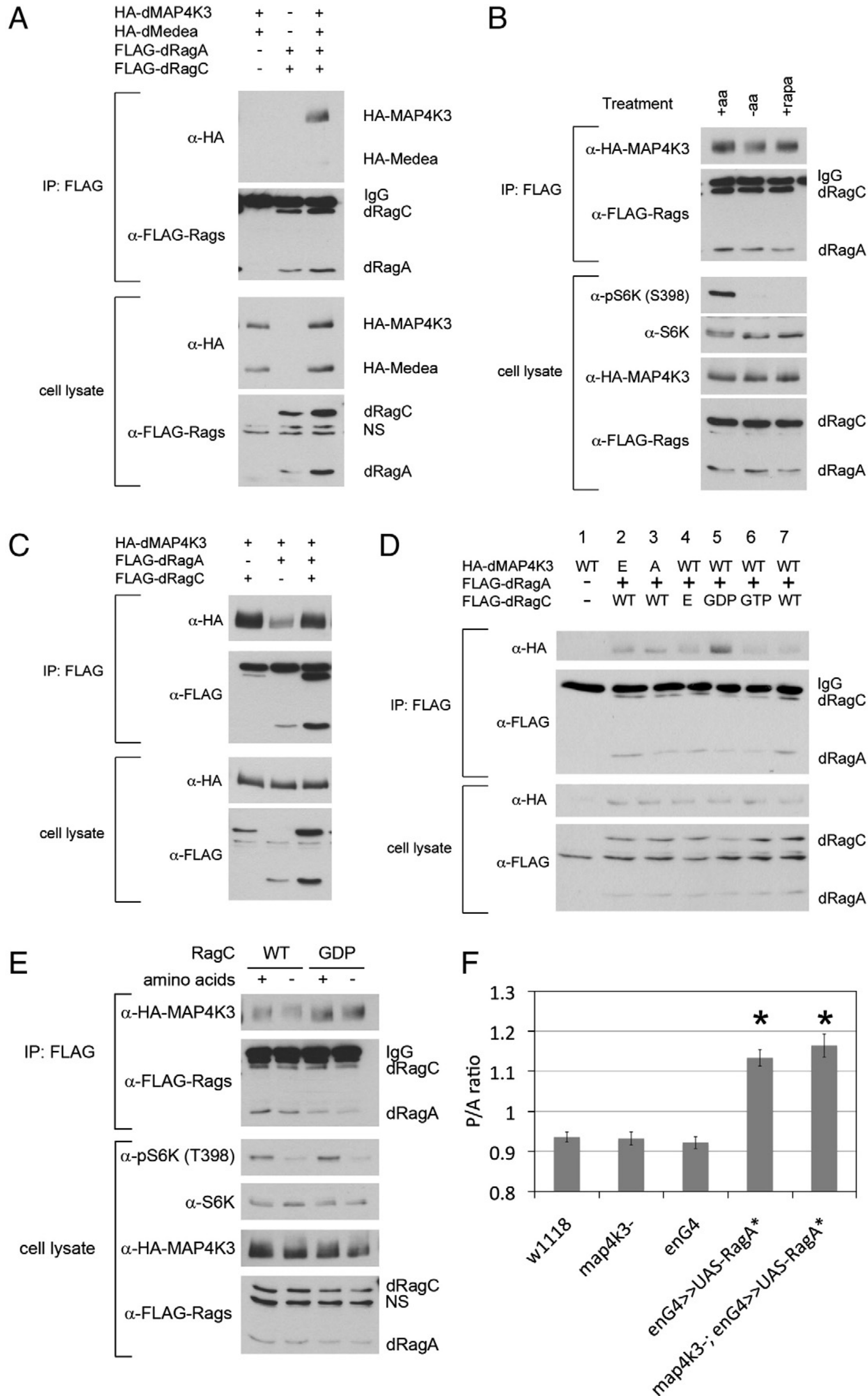


Fig. 5. Effects of amino acids on MAP4K3 mutant phenotypes. (A, B) Pupation curves for control (dark graphs) and map4k3 mutant flies (light graphs). Flies were grown in parallel on (A) normal laboratory food or (B) food containing only 1/5th the normal amount of yeast extract and soy meal, the two principle amino-acid containing components. Error bars indicate standard deviation. (C) Protein extracts from control or MAP4K3 mutant pre-wandering third-instar female larvae, probed for phospho- and total S6K, and ERK as a loading control.

In response to amino acid deprivation, the activation state of both the Rag proteins and of MAP4K3 changes: Rag proteins change their GDP/GTP loading (Kim et al., 2008; Sancak et al., 2008) whereas MAP4K3 becomes dephosphorylated (Yan et al., 2010). To study in more detail whether these events affect binding between MAP4K3 and the RagA/C complex, we first asked whether MAP4K3 binds preferentially to RagA



or to RagC. S2 cells were co-transfected with HA-MAP4K3 and either FLAG-RagA or FLAG-RagC, or both combined. Notably, the binding to RagC was significantly stronger than the binding to RagA (Fig. 6C, lanes 1 and 2) suggesting that binding of MAP4K3 to the RagA/C complex is mediated via binding to RagC. We then asked whether the activation state of MAP4K3 or RagC affects binding of MAP4K3 to the RagA/C complex. To this end, we generated a panel of mutations in either MAP4K3 or RagC. Lamb and colleagues have reported that the phosphorylation state of Ser170 of hMAP4K3 (Ser180 in Drosophila) is dependent on the presence of amino acids (personal communication, in press). Therefore, we generated dMAP4K3 variants in which Ser180 was mutated to either non-phosphorylatable alanine, or to glutamic acid mimicking the phosphorylated state. Neither mutation, however, affected binding of MAP4K3 to the RagA/C complex (Fig. 6D, lanes 2, 3 and 7). We also generated RagC variants that were either locked in the GDP or GTP state, or had a phospho-mimicking mutation on Ser388 (S388E), as RagC is reported to be phosphorylated at this site in vivo (http://www.phosphopep.org). Although mutation of Ser388 did not alter binding between MAP4K3 and the RagA/C complex, locking RagC into the GDP state strongly increased binding to MAP4K3 (Fig. 6D, lanes 4–7). Since amino acid availability regulates the GDP/GTP load of Rag proteins (Kim et al., 2008; Sancak et al., 2008), we asked whether amino acids regulate MAP4K3/Rag binding via modulation of the Rag GDP/GTP load. Whereas removal of amino acids caused a slight reduction in binding of MAP4K3 to the RagA/RagC(WT) complex (Fig. 6E, lanes 1–2), amino acid removal no longer had an effect when RagC was locked in the GDP state (Fig. 6E, lanes 3–4). This suggests that amino acids regulate MAP4K3/Rag binding by modulating the GDP/GTP load of the Rag proteins.

These biochemical data suggest that MAP4K3 and the Rag GTPases might work together in a signaling pathway mediating amino acid signaling to TORC1. They do not, however, shed light on which component is upstream of the other. To this end, we tested in vivo genetic epistasis between MAP4K3 and RagA. Overexpression of constitutively active RagA (Q61L) in Drosophila leads to tissue overgrowth, as would be expected from activation of TORC1 (Kim et al., 2008). Expression of RagA(Q61L) in the posterior compartment of the wing with engrailed-GAL4 caused an increase in the size of this compartment relative to the anterior (Fig. 6F, columns 1–4). When RagA(Q61L) was expressed in the posterior compartment in a map4k3-mutant background, it was still able to induce tissue overgrowth (Fig. 6F, column 5), indicating that MAP4K3 is not downstream of RagA.

Discussion

MAP4K3 mutant flies display phenotypes typical of reduced TORC1 activity and reduced nutrient availability

Recent reports have shown that not all components identified in cell culture as regulators of TORC1 activity also affect TORC1 *in vivo* in an animal model (Juhasz et al., 2008). The purpose of our study was two-fold: (1) to analyze whether MAP4K3 regulates TORC1 activity in vivo in the fly, and (2) to study the physiological consequences for the organism when the ability of TORC1 to sense amino acids is impaired.

We present biochemical evidence that TORC1 activity is reduced in *MAP4K3* mutant animals, consistent with published cell-culture data showing that MAP4K3 is required for full TORC1 activation (Findlay et al., 2007). Furthermore, *MAP4K3* mutants have defects typical of reduced TORC1 activity. They are delayed in their development due to a reduced rate of growth. They eventually pupate leading to adults of reduced size and their tissues are comprised of cells that are smaller than normal. Furthermore, *MAP4K3* mutants have significantly reduced triglyceride stores compared to controls. These physiological effects are similar to the phenotypes observed with mutants for other regulators of TOR, such as Melted. Melted mutant flies are also 10% smaller than controls and are significantly leaner (Teleman et al., 2005b).

As a whole, the *MAP4K3*- mutant phenotypes emulate the physiological effects observed when flies are grown on conditions of limiting food. When wildtype larvae are put on a low-nutrient diet, they are delayed in pupation and yield animals of small size that are lean (KH unpublished). Thus loss of MAP4K3 activity phenocopies a reduced nutrient environment, consistent with MAP4K3 playing a role in the ability of animals to sense their nutrient conditions. This suggests the ability of TORC1 to sense amino acids is most important when nutrient conditions are rich, allowing animals to accelerate their growth accordingly. In contrast, on a low-nutrient diet, control and MAP4K3 mutant flies grow equally slowly (Figs. 3A', 5B) consistent with TOR activity being low in both groups. This parallels nicely the results reported in cell culture by (Findlay et al., 2007): In the absence of amino acids, both control and MAP4K3 knockdown cells have low TOR activity whereas in the presence of amino acids, TOR is activated strongly in control cells but only weakly in MAP4K3 knockdown cells.

Unexpectedly, we found that *MAP4K3* mutant animals are viable, although they have an elevated mortality rate compared to controls. This suggests that the amino acid sensing pathway might only modulate TORC1 activity. If TORC1 activity were completely blunted in MAP4K3 mutants, the animals would be dead, as is the case for TOR or Rheb mutants. Consistent with this, we observe residual TORC1 activity in MAP4K3 mutants, as detected by phosphorylation of the TORC1 targets S6K and 4EBP. This also parallels results from cell culture. The results presented in Findlay et al., 2007 are obtained with cells starved of serum and consequently of insulin signaling. In the presence of insulin signaling, which resembles the physiological situation more closely, MAP4K3 mutant cells still retain residual TOR activity (unpublished), similar to what we observe in vivo here. Consistent with these findings, we observed that the Rheb expression is able to drive tissue growth also in the absence of MAP4K3 (Supplementary Fig. 3).

MAP4K3 binds Rag GTPases

Both MAP4K3 and the Rag GTPases have recently been shown to be required for amino acids to stimulate TORC1 activity. While studying dMAP4K3, we noticed that dMAP4K3 binds physically to the Rag GTPases, suggesting they might act together as components of a single

Fig. 6. dMAP4K3 interacts physically with dRagA and dRagC. (A) dMAP4K3 co-immunoprecipitates with the dRagA/dRagC complex. S2 cells were transfected with constructs expressing HA-dMAP4K3, HA-Medea, FLAG-dRagA, and FLAG-dRagC as indicated. Cell lysates and FLAG-tag immunoprecipitates were analyzed by immunoblotting against HA- and FLAG-tags. HA-MAP4K3 but not HA-Medea co-immunoprecipitates with FLAG-Rags (lane 3). HA-MAP4K3 does not precipitate in the absence of the FLAG-Rags (lane 1). "IgG" label marks the heavy chain of the anti-FLAG antibody, which runs slightly above dRagC. "NS" marks a non-specific band recognized by the anti-FLAG antibody in S2 cell lysates. (B) Effect of rapamycin and amino-acid withdrawal on binding between MAP4K3 and RagA/C. Cells and lysates were treated as in (A) except cells were treated with 20 nM rapamycin or treated with Schneider's medium lacking amino acids for 1 h prior to lysis. (C) dMAP4K3 binds dRagC more strongly than dRagA. Cells and lysates were treated as in (A). MAP4K3 can be detected more strongly in the FLAG immunoprecipitate when FLAG-RagC was expressed compared to FLAG-RagA. (D) Effect of various MAP4K3 and RagC mutations on binding between MAP4K3 and the RagA/C complex. MAP4K3 binds best to RagA/C complex when RagC is locked in the GDP conformation. Constructs used are wildtype MAP4K3 ("WT") or MAP4K3 containing a S180A ("A") or S180E ("E") mutation, as well as wildtype RagC ("WT"), RagC containing a S388E mutation ("E"), RagC locked in the GDP conformation (S54N, "GDP") or RagC locked in the GTP conformation (Q99L, "GTP"). (E) Binding of MAP4K3 to RagA/C is no longer sensitive to amino acid availability when RagC is locked in the GDP state. Cells expressing wildtype MAP4K3 together with RagA and either RagC(WT) or constitutively active RagC(Q61L) were treated as in (B). (F) Constitutively active RagA can induce tissue growth also in the absence of MAP4K3. Wing posterior compartment area normalized to anterior compartment area plotted for control w1118, map4k3 mutant, and engrailed-GAL4 flies (enG4), as well as flies expressing constitutively active RagA(Q61L) either in the wildtype background (enG4>>UAS-RagA*) or in the map4k3 mutant background (map4k3-; enG4>>UAS-RagA*). Error bars indicate standard deviation. *t-test < 10–11 relative to w1118.

signaling pathway. This interaction is likely specific for several reasons: (1) we could not detect binding of MAP4K3 to another GTPase, Rheb, (2) binding of MAP4K3 to the RagA/C complex was significantly stronger than binding of an unrelated HA-tagged protein, HA-medea (3) MAP4K3 bound FLAG-RagC significantly stronger than FLAG-RagA showing that MAP4K3 distinguishes between two Rag proteins and (4) binding of MAP4K3 to RagC depended on its GDP/GTP state.

Further studies will be required to test whether this interaction is important for TORC1 to sense amino acids. Our data suggest that MAP4K3 might be functioning upstream of the Rag GTPases, and not downstream since activated RagA does not require MAP4K3 to promote tissue growth in vivo (Fig. 6F). This raises the possibility that the Rag GTPases may be substrates for MAP4K3 phosphorylation. Indeed, RagC is phosphorylated in vivo in Kc167 cells on Ser388 (http://www.phosphopep.org). If MAP4K3 were to phosphorylate RagC, this would provide a mechanism for regulation of the Rag GTPases, which to date is mysterious. Work in the near future should shed further light on this issue.

In summary, we have characterized the physiological function of MAP4K3 in Drosophila, and shown that it modulates TORC1 activity, tissue growth and lipid metabolism in the animal. Physical interaction data hints at a possible link between MAP4K3 and the Rag GTPases. We show that the organismal function of amino acid sensing by TORC1 is mainly required to spur growth when nutrient conditions are rich.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.04.027.

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