Drosophila RNA polymerase III repressor Maf1 controls body size and developmental timing by modulating $tRNA_i^{Met}$ synthesis and systemic insulin signaling

Elizabeth J. Rideout, Lynne Marshall, and Savraj S. Grewal[1]

Department of Biochemistry and Molecular Biology, Clark H. Smith Brain Tumour Centre, Southern Alberta Cancer Research Institute, University of Calgary, Calgary, AB, Canada T2N 4N1

Edited by Lynn M. Riddiford, Howard Hughes Medical Institute, Janelia Farm Research Campus, Ashburn, VA, and approved December 2, 2011 (received for review August 15, 2011)

The target-of-rapamycin pathway couples nutrient availability with tissue and organismal growth in metazoans. The key effectors underlying this growth are, however, unclear. Here we show that Maf1, a repressor of RNA polymerase III-dependent tRNA transcription, is an important mediator of nutrient-dependent growth in *Drosophila*. We find nutrients promote tRNA synthesis during larval development by inhibiting Maf1. Genetic inhibition of Maf1 accelerates development and increases body size. These phenotypes are due to a non–cell-autonomous effect of Maf1 inhibition in the fat body, the main larval endocrine organ. Inhibiting Maf1 in the fat body increases growth by promoting the expression of brain-derived insulin-like peptides and consequently enhanced systemic insulin signaling. Remarkably, the effects of Maf1 inhibition are reproduced in flies carrying one extra copy of the initiator methionine tRNA, $tRNA_i^{Met}$. These findings suggest the stimulation of $tRNA_i^{Met}$ synthesis via inhibition of dMaf1 is limiting for nutrition-dependent growth during development.

metabolism | physiology | endocrinology | amino acids | adipose tissue

How growth and size are controlled during animal development is an important question in biology. Several families of conserved cell–cell signaling pathways (e.g., Wnts, Hedgehog, Notch, BMPs, and receptor tyrosine kinase signaling) regulate organ size by controlling cell growth, proliferation, and survival (1). In addition, environmental factors such as nutrients, oxygen, and temperature influence tissue and organismal growth during development (1). The conserved target of rapamycin (TOR) kinase is perhaps the best-understood nutrient-dependent regulator of cell metabolism and growth in animals (2). The complex signaling network that couples extracellular nutrients to the activation of TOR has been extensively studied in simple systems such as yeast and in vitro cultured cells (3). However, it is studies in model organisms, most notably *Drosophila*, that have begun to reveal the role for nutrient/TOR signaling in the control of tissue and organismal growth (4). During a 4-d period of growth, *Drosophila* larvae increase in mass ∼200-fold. This growth is dependent on nutrition—in particular, dietary protein. Because neither pupae nor adults grow, final body size is determined by both the rate of larval growth and the duration of the larval period (5). TOR signaling regulates body size by modulating these parameters of growth. For example, TOR signaling is cell-autonomously required for nutrition-dependent growth in all larval tissues (6, 7); loss of TOR function in individual cells or tissues leads to a subsequent reduction in cell size or tissue mass (6, 7). Importantly, modulation of TOR activity in specific tissues can also influence overall body size by exerting non–cell-autonomous, humoral effects on organismal growth and developmental timing (8, 9). For example, TOR signaling in the fat body couples dietary nutrients to systemic insulin signaling and body growth (9). Thus, in nutrient-rich conditions, amino acid import into fat cells promotes TOR activity, leading to the relay of a secreted factor(s) that triggers the release of several *Drosophila* insulin-like peptides (dILPs) from neurosecretory cells (NSC) in the brain (9). These dILPs then circulate throughout the animal and promote growth in all tissues. Similarly, TOR signaling in the prothoracic gland influences body size by modulating the release of the insect steroid hormone ecdysone, which controls the timing of pupation, and hence the duration of the larval period (8).

An important, but unresolved, issue concerns the identity of the key downstream effectors of nutrient/TOR-dependent animal growth. Stimulation of protein synthesis has been widely proposed as a growth-promoting output of the TOR pathway, based largely on studies in yeast and mammalian cell culture (2, 3, 10). The prevailing model is that nutrient/TOR signaling promotes protein synthesis via regulation of mRNA translation initiation. In particular, phosphorylation and inhibition of 4E-binding protein (4E-BP), a conserved repressor of eukaryotic initiation factor 4E (eIF4E), is thought to mediate many of TOR's effects on translation (3, 10). However, genetic experiments in flies, worms, and mice suggest that 4E-BP has limited effects on tissue and organismal growth (11). Another mechanism by which TOR controls protein synthesis is by regulating the abundance of small noncoding RNAs, such as tRNA and 5S rRNA (12, 13). These RNAs are transcribed by RNA polymerase III (Pol III) and are essential for ribosome synthesis and mRNA translation (14). Recent reports in yeast and mammalian cell culture showed that nutrients and TOR signaling promote tRNA synthesis by inhibiting Maf1, a conserved repressor of Pol III-dependent tRNA transcription (15–17). However, the significance of the regulation of tRNA synthesis by Maf1 in the context of cell, tissue, and organismal growth during animal development has not been explored.

Here we identify a role for *Drosophila* Maf1 (*dMaf1*) as a regulator of nutrient-dependent growth and development. Importantly, we show that *dMaf1* influences growth primarily in a non–cell-autonomous manner. During normal growth, when nutrients are abundant, inhibition of *dMaf1* specifically in the fat body promotes tRNA synthesis, leading to increased organismal growth by stimulating systemic insulin signaling. Significantly, we identify a single transcript, the initiator methionine tRNA ($tRNA_i^{Met}$), as the primary trigger of organismal growth in this context.

Author contributions: E.J.R. and S.S.G. designed research; E.J.R., L.M., and S.S.G. performed research; E.J.R. contributed new reagents/analytic tools; E.J.R. analyzed data; and E.J.R. and S.S.G. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence should be addressed. E-mail: grewalss@ucalgary.ca.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1113311109/-/DCSupplemental.

Results

Inhibition of *dMaf1* Increases tRNA Synthesis, Accelerates Development, and Augments Body Size. We first confirmed that *Drosophila Maf1* (*dMaf1*, CG40196) functions as a repressor of tRNA synthesis in vivo in larvae. Ubiquitous expression of a *UAS-dMaf1 RNAi* construct in larvae throughout development using the *daughterless-GAL4* (*da>*) driver led to a reduction in dMaf1 protein levels (Fig. S1*A*) and a global increase in tRNA levels compared with controls (Fig. 1*A*). This result is consistent with a repressor function for dMaf1. Other Pol III-dependent transcripts were unaffected, no effect was observed on tRNA synthesis with the *UAS-dMaf1 RNAi* line alone, and the increase in tRNA synthesis was reversed by overexpression of a *UAS-dMaf1* transgene (Fig. S1 *B–D*). Interestingly, we observed a marked increase in the larval growth rate when we inhibited *dMaf1*; the *da>dMaf1 RNAi* larvae progressed from egg laying to pupation ~24 h sooner than control larvae (Fig. 1*B*). Because body size is determined by growth during the larval stage (5), pupal volume measurements provide an accurate index of both larval growth and adult body size. We observed a significant increase in pupal volume in *da>dMaf1 RNAi* pupae compared with controls (Fig. 1 *C* and *D*). This increase in size was reproduced in another independent insertion of the *dMaf1 RNAi* transgene, as well as in two independent insertions of a *dMaf1 RNAi* transgene targeting an overlapping, but smaller, region of *dMaf1* (Fig. S1*E*). Moreover, the increase in body size was reversed by overexpression of *dMaf1*, further confirming the specificity of the *dMaf1 RNAi* transgene (Fig. S1*F*). Overexpression of *dMaf1* alone did not, however, have a dominant effect on tRNA synthesis or body size (Fig. S2). Thus, specific inhibition of *dMaf1* is sufficient to augment tRNA levels and to promote an increase in organismal growth and the rate of development.

Elevated Synthesis of Single tRNA (Initiator Methionine) Is Sufficient to Drive Growth. As a repressor of tRNA synthesis, dMaf1 regulates the synthesis of all tRNAs. Any of these transcripts individually or in combination could potentially mediate the observed increase in larval growth rate and body size upon *dMaf1* inhibition. A previous report, however, demonstrated that elevated levels of $tRNA_i^{Met}$ alone enhanced proliferation in cultured mammalian fibroblasts (18). We therefore hypothesized that levels of $tRNA_i^{Met}$ may be the limiting factor for the stimulation of growth and body size following inhibition of *dMaf1*. To test this hypothesis we made transgenic flies with a single *P*-element insertion, where the *P* element contains one extra copy of the $tRNA_i^{Met}$ gene, which we will refer to as $P\{tRNA_i^{Met}\}$ flies. Levels of $tRNA_i^{Met}$, but not other tRNAs, were significantly elevated in $P\{tRNA_i^{Met}\}$ larvae (Fig. 2*A*). Strikingly, when we measured the development of these larvae we saw an accelerated growth rate and increased final size (Fig. 2 *B–D*). We observed similar increases in growth in two independent $P\{tRNA_i^{Met}\}$ transgenic lines (Fig. S3 *A* and *B*). In contrast, no increase in growth was observed in transgenic flies carrying a *P* element with an extra copy of other tRNAs, such as tRNA elongator methionine ($P\{tRNA_e^{Met}\}$), tRNA arginine ($P\{tRNA^{Arg}\}$) (Fig. 2*D* and Fig. S3*C*), or tRNA alanine ($P\{tRNA^{Ala}\}$) (Fig. S3*C*). Thus, increased synthesis of only one tRNA, $tRNA_i^{Met}$, can phenocopy the effects of *dMaf1* inhibition to drive growth and development in vivo.

Inhibition of *dMaf1* and Elevated $tRNA_i^{Met}$ Synthesis Promotes Growth by Stimulating Protein Synthesis. It is remarkable that simply increasing the levels of a single tRNA can enhance organismal growth. However, $tRNA_i^{Met}$ plays a unique role among tRNAs in eukaryotic cells. It is assembled into a ternary complex along with eIF2 and GTP (19). This complex then associates with the 40S ribosome and other eIFs to trigger ribosome scanning of mRNAs and to initiate translation, and so it is possible that

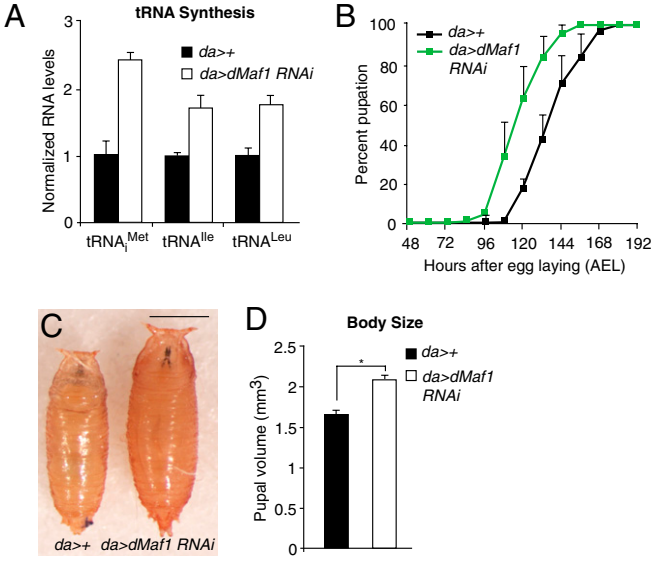

Fig. 1. Loss of *dMaf1* increases growth rate and body size. (*A*) Levels of $tRNA_i^{Met}$, $tRNA^{Ile}$, and $tRNA^{Leu}$ were significantly elevated in *da>dMaf1 RNAi* larvae compared with *da>+* controls when measured by quantitative real-time PCR ($P = 7.3 \times 10^{-6}$; 0.0002 and 0.001, respectively; Student's *t* test). Data were normalized to β-tubulin. (*B*) Time to pupation in *da>dMaf1 RNAi* larvae was decreased by 24 h compared with *da>+* controls ($n > 200$). (*C* and *D*) Pupal volume in *da>dMaf1 RNAi* flies was significantly greater than *da>+* controls ($P = 2 \times 10^{-12}$, Student's *t* test, $n = 58$). (Scale bar: *C*, 1 mm.) Error bars represent SEM.

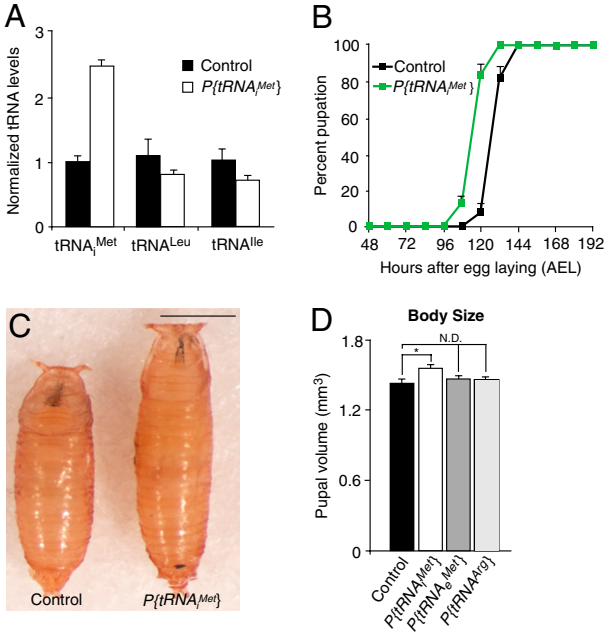

Fig. 2. Increased synthesis of $tRNA_i^{Met}$ stimulates growth. (*A*) Levels of $tRNA_i^{Met}$ and not other tRNAs were significantly elevated in $P\{tRNA_i^{Met}\}$ larvae compared with w^{1118} controls when measured by quantitative real-time PCR ($P = 0.0002$; 0.36, and 0.21, respectively, Student's *t* test). Data were normalized to β-tubulin. (*B*) The time to pupation in $P\{tRNA_i^{Met}\}$ larvae was decreased compared with w^{1118} controls ($n > 200$). (*C*) Body size of $P\{tRNA_i^{Met}\}$ pupae is larger than controls. (Scale bar: 1 mm.) (*D*) Volume of $P\{tRNA_i^{Met}\}$ pupae ($P = 1.3 \times 10^{-5}$), not $p\{tRNA_e^{Met}\}$ or $P\{tRNA^{Arg}\}$ ($P = 0.18$ and 0.07, respectively), was significantly greater than w^{1118} controls (Student's *t* test, $n = 90$). Error bars represent SEM. N.D., not significantly different.

$tRNA_i^{Met}$ levels are limiting for protein synthesis. Indeed, we found that $P\{tRNA_i^{Met}\}$ larvae had a significantly higher amount of total protein per larva than controls (Fig. 3*A*). Using polysome gradient centrifugation, we also observed a 40% increase in total RNA contained in the polysome fraction in $P\{tRNA_i^{Met}\}$ larvae compared with control larvae (Fig. 3*B* and Fig. S4 *A* and *B*), consistent with elevated mRNA translation. Finally, we found that the increase in body size in both *da>dMaf1 RNAi* and $P\{tRNA_i^{Met}\}$ pupae was suppressed in flies heterozygous for a loss-of-function allele of ribosomal protein S3 (RPS3) that reduces translation capacity (Fig. 3 *C* and *D*). Together, these results suggest that elevated $tRNA_i^{Met}$ synthesis augments growth by enhancing protein synthesis. Prevailing models implicate regulation of eIFs as the limiting factor for stimulation of cellular mRNA translation and growth (19). Our findings, however, demonstrate that increasing $tRNA_i^{Met}$ alone is sufficient, revealing an additional mechanism to control protein synthesis and growth during animal development.

Inhibition of *dMaf1* Has a Limited Effect on Cell-Autonomous Growth. A previous study showed that the stimulation of protein synthesis promoted the proliferation of cultured mammalian cells (18). Thus, the increase in body size caused by inhibition of *dMaf1* may have resulted from cell-autonomous increases in cell size and/or cell number in all developing larval tissues.

We therefore explored whether stimulating tRNA synthesis could mediate cell-autonomous effects on growth in mitotic (imaginal discs, central nervous system) and/or polyploid (muscle, fat body, gut) tissues in the developing larvae. We first examined cell size and proliferation in the adult wing, which develops from the larval wing imaginal disc, a mitotic tissue. Because $tRNA_i^{Met}$, like all tRNA genes, contains an internal Pol III promoter that precludes GAL4-mediated tissue-specific expression, we stimulated tRNA synthesis by expressing the *dMaf1 RNAi* transgene. When we restricted expression of the *dMaf1 RNAi* transgene to all of the cells of the developing wing posterior compartment (using *engrailed-GAL4, en>*), we observed an increase in compartment size and thus a larger posterior:anterior compartment size ratio (Fig. S5*A*). We also found a significant increase in cell number in the posterior compartment in *en>dMaf1 RNAi* flies (Fig. S5*B*), consistent with an increase in the rate of cell division. In contrast, posterior compartment cell size was unchanged in both adult (Fig. S5*C*) and larval *en>dMaf1 RNAi* wing cells (Fig. S5*D*). Inhibition of *dMaf1* therefore leads to a modest (~7%) increase in tissue mass in mitotic tissues by stimulating cell growth and proliferation. We next examined cell size in the polyploid cells of the larval fat body. We used the *flp-out* system to generate random mosaic clones of *dMaf1 RNAi*-expressing cells in the fat body (20). We found that knockdown of *dMaf1* had no significant effect on cell size (Fig. S5 *E* and *F*). Together these data suggest that inhibition of *dMaf1* has only a modest effect on cell growth and proliferation in mitotic tissues, and no effect on cell growth in endoreplicating tissues. Thus, the increased body size we observed with ubiquitous inhibition of *dMaf1* cannot be due solely to a cell-autonomous stimulation of growth and proliferation in developing organs. Rather, our results suggest that additional systemic growth mechanisms must contribute to this increased growth. We present evidence below for such a mechanism involving the fat body, a key larval endocrine organ.

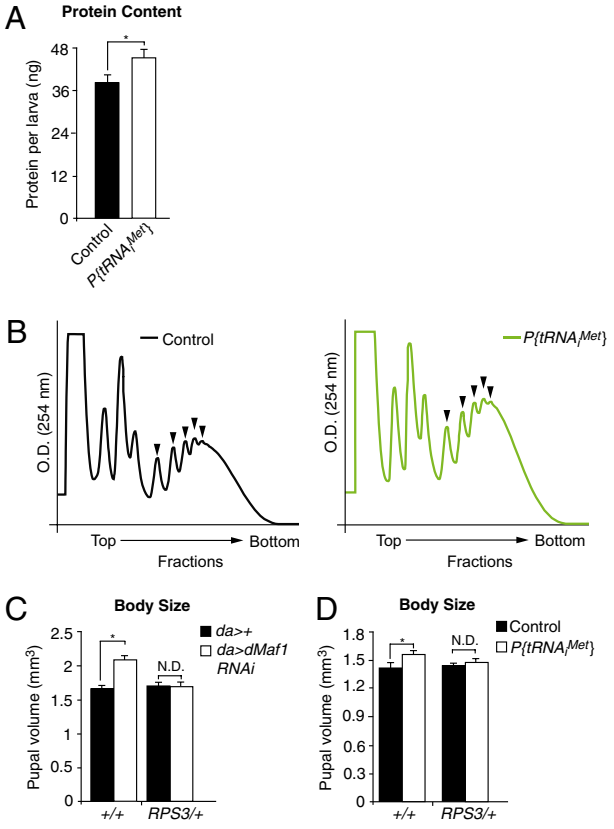

Fig. 3. Elevated tRNA synthesis stimulates mRNA translation. (*A*) Total protein per larva was significantly increased in $P\{tRNA_i^{Met}\}$ larvae compared with w^{1118} controls when normalized for DNA ($P = 0.008$; Student's *t* test). (*B*) Representative polysome profiles from w^{1118} (black trace) and $P\{tRNA_i^{Met}\}$ larvae (green trace). Polysome peaks (arrowheads) in $P\{tRNA_i^{Met}\}$ larvae were higher compared with controls, suggesting translation was increased, and percent of total RNA contained in the polysome fraction was significantly increased in $P\{tRNA_i^{Met}\}$ larvae ($P = 0.043$, Student's *t* test; see Fig. S4*A* for graph). Increased size in (*C*) *da>dMaf1 RNAi* ($P = 6.1 \times 10^{-10}$; Student's *t* test) and (*D*) $P\{tRNA_i^{Met}\}$ pupae ($P = 6.3 \times 10^{-5}$, Student's *t* test) was suppressed when heterozygous for a null allele of ribosomal protein S3 (RPS3; $P = 0.44$ and 0.115, respectively, Student's *t* test, $n > 80$). Error bars represent SEM.

Nutrient Availability Inhibits *dMaf1* in the Fat Body to Promote tRNA Synthesis and Stimulate Organismal Growth. The larval fat body functions as both a sensor of nutritional status and as an endocrine organ (21). In nutrient-rich conditions, the fat body signals to the brain to stimulate dILP release and promote systemic insulin signaling and growth (9). A major trigger for this fat-to-brain signal is nutrient/TOR signaling and protein synthesis in fat cells (22). We therefore hypothesized that the organismal effects of *dMaf1* inhibition may arise from loss of *dMaf1* function specifically in the fat body. In particular, we explored whether nutrient availability might stimulate systemic growth by inhibiting *dMaf1* in fat cells to promote tRNA synthesis. We first found that levels of tRNA synthesis in the fat body of feeding larvae were significantly higher than in fat bodies from larvae starved of amino acids for 24 h (Fig. 4*A*). Levels of tRNA synthesis were also decreased upon starvation in the muscle (Fig. S6*A*). Furthermore, we found that in feeding larvae, silencing of *dMaf1* specifically in the fat body using the *cg-GAL4* driver (*cg>*) led to elevated levels of tRNAs in fat body cells compared with control fat bodies (Fig. 4*B* and Fig. S6*B*). A similar result was obtained with another *dMaf1 RNAi* line (Fig. S6*C*). Moreover, upon starvation for dietary proteins, when levels of tRNA synthesis were normally suppressed in control larvae, there was no change in the levels of tRNA synthesis in the fat body of *cg>dMaf1 RNAi* larvae (Fig. 4*B* and Fig. S6 *B* and *C*). To further link the regulation of tRNA synthesis with upstream nutrient-sensing pathways, we fed larvae rapamycin, a specific TOR inhibitor. Inhibiting TOR in this manner led to a significant decrease in tRNA synthesis in the fat body of control *cg>+* animals (Fig. 4*C*). In contrast, tRNA synthesis the fat bodies of *cg>dMaf1*

RNAi larvae was unaffected following 48 h of rapamycin treatment (Fig. 4*C*). These data demonstrate that nutrient/TOR signaling normally promotes tRNA synthesis in the fat body via *dMaf1* inhibition. We therefore asked whether genetic inhibition of *dMaf1* in the fat body could influence organismal growth and final size. When we expressed the *dMaf1 RNAi* transgene using *cg-GAL4* (*cg>*), we phenocopied the increased larval growth rate and body size caused by a ubiquitous increase in tRNA synthesis. Thus, *cg>dMaf1 RNAi* larvae progressed from egg laying to pupation ~16 h faster than control larvae (Fig. 4*D*). Similar results were obtained using a second fat body-specific GAL4 driver, *ppl-GAL4* (Fig. 4*E*), but not with several other tissue-specific drivers (Fig. 4 *F* and *G* and Fig. S7 *A* and *B*). We also found that pupal volume was significantly increased in *cg>dMaf1 RNAi* animals compared with controls (Fig. S7*C*). Together, these results support our hypothesis that when nutrients are abundant, amino acid uptake into the fat body stimulates tRNA synthesis and consequently organismal growth via inhibition of *dMaf1*.

Loss of *dMaf1* in the Fat Body Stimulates Systemic Insulin Signaling. Previous reports have shown that fat body-mediated effects on organismal growth occur via stimulation of systemic insulin signaling (9, 22). We therefore tested whether inhibition of *dMaf1* in the fat body led to alterations in systemic insulin signaling. dILP 2 and dILP 5 are two brain-derived growth-promoting dILPs whose expression and release from the NSCs is increased by the fat-to-brain signal (9, 23, 24). When we expressed *dMaf1 RNAi* specifically in the fat body (*cg>dMaf1 RNAi*), we found elevated transcript levels of both *dilp2* and *dilp5* in peripheral tissues of these larvae compared with controls (Fig. 5 *A* and *B*). This increase in *dilp2* mRNA is due at least in part to increased transcript levels in the brain (Fig. S7*D*). When insulin signaling is high, the PI3K-Akt pathway inhibits the FOXO transcription factor, leading to reduced levels of FOXO transcriptional targets, such as *Drosophila insulin receptor* (*dInR*) (25). Therefore,

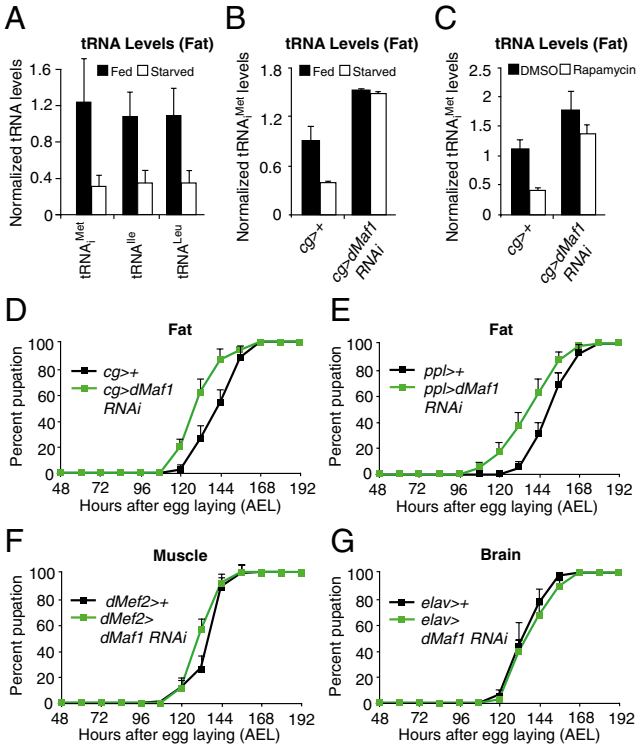

Fig. 4. Loss of *dMaf1* in the fat stimulates growth via a non–cell-autonomous mechanism. (*A*) Following 24-h amino acid starvation, levels of $tRNA_i^{Met}$, $tRNA^{Ile}$, and $tRNA^{Leu}$ synthesis were significantly decreased in the fat body when measured by quantitative real-time PCR ($P = 0.0007$, 3×10^{-5}, 4×10^{-6}, respectively). Data were normalized to β-tubulin. (*B*) Levels of $tRNA_i^{Met}$ synthesis were significantly elevated in fat bodies of *cg>dMaf1 RNAi* larvae compared with *cg>+* controls ($P = 0.01$, Student's *t* test). Levels of tRNA synthesis remained significantly elevated in *cg>dMaf1 RNAi* larvae compared with *cg>+* controls following 24-h starvation in 20% sucrose in PBS ($P = 5 \times 10^{-7}$, Student's *t* test). (*C*) Levels of $tRNA_i^{Met}$ synthesis were significantly elevated in fat bodies of *cg>dMaf1 RNAi* larvae fed on DMSO for 48 h compared with *cg>+* controls ($P = 0.017$, Student's *t* test). Although levels of $tRNA_i^{Met}$ synthesis were significantly decreased following rapamycin treatment in *cg>+* fat bodies ($P = 6.4 \times 10^{-4}$, Student's *t* test), levels of $tRNA_i^{Met}$ synthesis in *cg>dMaf1 RNAi* fat bodies were unchanged ($P = 0.17$, Student's *t* test). (*D*–*G*) Time to pupation in larvae with (*D* and *E*) fat body-, (*F*) muscle-, or (*G*) neuron-specific loss of *dMaf1* ($n > 400$). Error bars represent SEM.

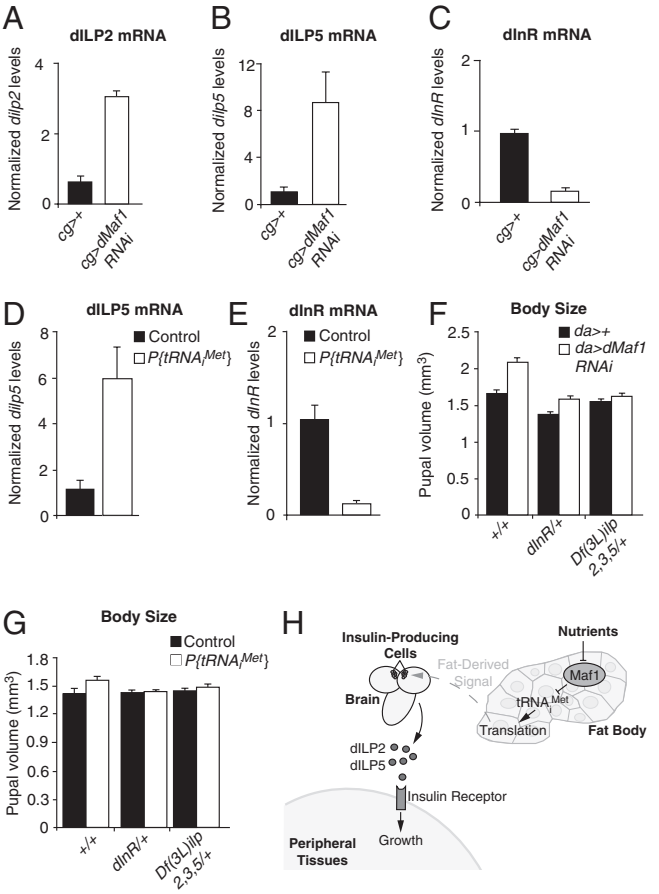

Fig. 5. tRNA synthesis in the fat body stimulates organismal growth by promoting systemic insulin signaling. *dilp2* (*A*) and *dilp5* (*B*) transcripts were elevated in *cg>dMaf1 RNAi* carcasses stripped of fat tissue compared with controls when measured by quantitative real-time PCR ($P = 0.013$ and 0.005, respectively, Student's *t* test). Data were normalized to β-tubulin. (*C*) *dInR* transcripts were significantly lower in *cg>dMaf1 RNAi* carcasses compared with *cg>+* controls ($P = 8.1 \times 10^{-8}$, Student's *t* test). (*D* and *E*) Transcript levels of *dilp5* (*D*) were significantly elevated in *P{tRNA$_i^{Met}$}* larvae compared with w^{1118} controls ($P = 0.004$, Student's *t* test), whereas *dInR* mRNA (*E*) was significantly lower than controls ($P = 3.4 \times 10^{-9}$, Student's *t* test). (*F* and *G*) Increased size in *da>dMaf1 RNAi* (*F*) and *P{tRNA$_i^{Met}$}* pupae (*G*) was suppressed when heterozygous for a null mutation in *dInR*, or a deletion uncovering the *dilp2*, *-3*, and *-5* genes ($n > 80$ for all genotypes). Error bars represent SEM. (*H*) Loss of *dMaf1* function in the larval fat body stimulates tRNA synthesis in the fat body. High levels of tRNA promote translation in the fat, leading to the increased secretion of the fat-derived signal. This signal promotes insulin production and/or release from the insulin-producing cells in the brain, which promotes growth in all peripheral tissues.

measuring *dInR* mRNA levels is used as a readout of insulin signaling (9, 22). We found that *dInR* transcripts were significantly decreased in peripheral tissues of *cg>dMaf1 RNAi* larvae compared with control larvae (Fig. 5*C*), suggesting an increase in insulin-PI3K signaling. Importantly, we observed similar increases in both dILP levels and systemic insulin signaling in $P\{tRNA_i^{Met}\}$ transgenic larvae (Fig. 5 *D* and *E*). Finally, we found the increased body size in *da>Maf1 RNAi* and $P\{tRNA_i^{Met}\}$ flies observed was dependent on insulin signaling because, in both cases, the phenotype was suppressed in pupae heterozygous for either a null allele of *dInR* or for a chromosomal deficiency that deletes the *dilp2*, *-3*, and *-5* genes (Fig. 5 *F* and *G*). Together these data support a model in which nutrient-dependent inhibition of *dMaf1* in the fat body can promote systemic growth and body size by increasing both dILP expression and peripheral insulin signaling (Fig. 5*H*).

Discussion

In most animal model systems, TOR signaling couples nutrition to organismal growth. Nevertheless, the key metabolic effectors of TOR remain unclear. In our study, we identify the repression of dMaf1 as a downstream function of nutrients/TOR in the control of tissue and organismal growth. We demonstrate that nutrients promote tRNA synthesis during development by inhibiting *dMaf1*. This result is consistent with previous observations in yeast and cell culture experiments showing that nutrient/TOR signaling directly inhibits Maf1 to promote tRNA synthesis (15–17). By investigating the significance of this repression in a developing animal, however, we define a role for *dMaf1* as a repressor of tissue and organismal growth. Furthermore, we showed that simply increasing the synthesis of a single tRNA, $tRNA_i^{Met}$, could phenocopy the effect of *dMaf1* inhibition. Based on these findings, we therefore propose a model of growth control in which nutrient/TOR signaling inhibits dMaf1 to stimulate $tRNA_i^{Met}$ synthesis, ultimately driving development and promoting organismal growth (Fig. 5*H*).

The effect of elevated $tRNA_i^{Met}$ synthesis on body size was associated with increased mRNA translation and was reversed by genetically reducing protein synthesis. As discussed in the introduction, the prevailing view is that inhibition of 4E-BP is a key target of nutrient/TOR signaling in the control of mRNA translation and growth. 4E-BP normally functions to inhibit eIF4E. TOR signaling, in turn, reverses this inhibition. This mode of translational control has been well established in cell culture (19). However, neither inhibition of 4E-BP nor overexpression of eIF4E—manipulations that promote mRNA translation—stimulate growth or affect the rate of development in flies (7, 11, 26, 27). Instead, our data suggest that the availability of $tRNA_i^{Met}$, rather than the activity of translation initiation factors, normally limits translation and growth in vivo. It is unclear whether $tRNA_i^{Met}$ is limiting simply because the absolute cellular levels of $tRNA_i^{Met}$ are lower than other tRNAs. Given that $tRNA_i^{Met}$ has a unique function as the initiating tRNA for cap-dependent translation, however, it is possible that cells have evolved to make $tRNA_i^{Met}$ synthesis or availability a limiting step for stimulation of protein synthesis. In particular, we propose that levels of $tRNA_i^{Met}$ must be fine-tuned in response to growth cues, such as nutrition, to ensure appropriate rates of mRNA translation, metabolism, and growth. This notion is reminiscent of the transcriptional control of rRNA in *Escherichia coli*, where the levels of the initiating NTP are limiting for activation of transcription by extracellular nutrients (28). Given the universal role for $tRNA_i^{Met}$ in translation initiation, it is tempting to speculate that regulation of $tRNA_i^{Met}$ represents a common mechanism used by cell–cell signaling pathways, particularly those involved in determining organ size (e.g., BMPs, Wnts, Hedgehog) to control growth.

An important finding of our work is that *dMaf1* inhibition promotes growth and development due to a specific effect in the larval fat body. The fat body functions as both a nutrient sensor and endocrine organism during *Drosophila* development. In particular, amino acid import into fat cells, and subsequent activation of TOR, promotes dILP release from the brain via a fat-to-brain secreted signal (9, 29). Our findings suggest inhibition of *dMaf1* and increased $tRNA_i^{Met}$ in fat cells is a key mediator of this physiological response. Similar effects on systemic insulin signaling were seen when ecdysone signaling was inhibited in the fat body (22). In this case, the fat body effects were due to increased dMyc and thought to involve elevated levels of ribosome synthesis (22). Thus, we propose that both TOR/Maf1 and ecdysone/Myc influence fat-to-brain signaling by promoting mRNA translation. Together, these studies highlight the critical importance of translational control in the fat body in regulating organismal growth (9, 22). Given the emerging appreciation of the functional similarities between the larval fat body and mammalian liver and adipose tissue (21), we propose that stimulating $tRNA_i^{Met}$ synthesis and consequently mRNA translation in these tissues underlie the coordination of systemic physiology, metabolism, and growth in mammals.

How does an increase in levels of $tRNA_i^{Met}$, and consequently increased mRNA translation, drive growth? In principle, a global increase in protein synthesis, including synthesis of metabolic enzymes required for growth, could account for changes in body size. A growing literature suggests, however, that modulating protein synthesis has selective effects on mRNA translation. For example, in both yeast and mammalian cells, alterations in nutrient/TOR signaling lead to differential mRNA translation (30, 31). Thus, only a subset of mRNAs are up- or down-regulated based largely on properties of individual mRNAs such as mRNA length or the secondary structure of the 5′ UTR (32). This mechanism of translational control is also conserved in flies and worms (33, 34), where dietary restriction or modulation of insulin signaling promoted differential translation of genes important for growth, stress responses, and aging (33, 34). We therefore propose that the main consequence of increased $tRNA_i^{Met}$ synthesis in the fat body is enhanced translation of specific mRNAs, ultimately leading to increased fat-to-brain signaling and organismal growth. The nature of the secreted factor(s) that signals from the fat to brain is unclear. However, perhaps either the factor itself (if it is a peptide) or genes required to synthesize or release it are translationally regulated by nutrient-dependent signaling in the fat body.

As well as increasing body size, *dMaf1* inhibition and increased $tRNA_i^{Met}$ synthesis also accelerated development. In insects, release of the steroid hormone ecdysone from the prothoracic gland (PG) is the primary regulator of developmental timing. Premature release of ecdysone accelerates progression through larval stages, leading to precocious pupation and smaller-sized adults (35–37). In contrast, delayed ecdysone release slows progression through larval stages, leading to late pupation and larger adults (36, 37). Dietary nutrition is an important regulator of ecdysone release and, consequently, developmental timing (8, 35–37). For example, several studies have demonstrated that increased PI3K and TOR signaling, two key effectors of dietary nutrients, within the PG can stimulate the release of ecdysone (35, 36). Conversely, reducing PI3K or TOR signaling in the PG delays ecdysone release and pupation (8, 36, 37). Finally, a recent study showed that increased expression of dILP2 in neurosecretory cells led to premature release of ecdysone and a significant acceleration of development (38). We therefore propose that inhibition of *dMaf1* in the fat body accelerates development through effects on systemic insulin. This increase in insulin signaling would stimulate growth in all tissues and activate PI3K signaling in the PG to trigger ecdysone release and precocious pupation. Together these effects promote both an overall increase in larval growth rate coupled with a shortening of the period of larval development.

In conclusion, our study has identified inhibition of tRNA synthesis by dMaf1 as a limiting factor for nutrition/TOR-induced tissue and organismal growth. These findings have several interesting implications for human biology. For example, deregulation of tRNA synthesis is observed in nearly all tumors (14). Our data, in combination with the previous study in mammalian fibroblasts (18), indicate that elevated tRNA synthesis, rather than being a consequence of increased growth, may in some cases drive protein synthesis, cell growth, and proliferation—factors that may ultimately contribute to tumor progression in vivo. Further, we show that regulation of tRNA synthesis is a limiting factor for mRNA translation and systemic insulin signaling. Because deregulation of both of these processes is a common occurrence in many pathological contexts, such as cancer, diabetes, and aging (10, 39–41), future studies on the regulation of $tRNA_i^{Met}$ synthesis may provide valuable insights into disease processes and progression.

Methods

Fly Strains. The following fly stocks were used: *w*^{1118}, *UAS-GFP*, *en-GAL4, yw;; dInR*^{PZ}*/TM3,Sb*, *da-GAL4*, *cg-GAL4*, *ppl-GAL4*, *ey-GAL4*, *dMef2-GAL4*, *elav-GAL4*, *dilp2-GAL4*, *Df(3L)ilp2-3,5/TM3,Sb*, *RPS3/TM6B,Tb,Hu*, *hsflp*^{122}*;+;+*, and *w*;+;act >CD2>GAL4*.

Pupal Volume. Pupal volume was calculated as described in Delanoue et al. (22).

Quantitative RT-PCR. Total RNA was extracted from larval tissues using TRIzol (Invitrogen), and cDNA was synthesized using SuperScript II (Invitrogen) according to manufacturer's instructions.

Additional details of experimental procedures are provided in *SI Methods*.

ACKNOWLEDGMENTS. We thank S. Salgia for technical assistance; G. Moorhead for help and advice in generating the dMaf1 antibody; B. Edgar, G. Thomas, and P. Shen for providing fly stocks; and S. Goodwin, J.-C. Billeter, P. Mains, W. Brook, J. McGhee, E. Kurz, and two anonymous referees for helpful comments on the manuscript. The β-tubulin (E7) antibody developed by Michael Klymkowsky was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa Department of Biology (Iowa City, IA). This work was supported by Canadian Institutes of Health Research Operating Grant MOP-86622 and the Alberta Cancer Foundation (S.S.G.). E.J.R. was supported by postdoctoral fellowships from the Natural Sciences and Engineering Council of Canada, Alberta Innovates-Health Solutions, and the Canadian Institutes of Health Research Training Program in Genetics, Child Development and Health (Alberta Children's Hospital Research Institute for Child and Maternal Health). L.M. was supported by postdoctoral fellowships from Alberta Innovates-Health Solutions and Alberta Health Services (Alberta Cancer Foundation/Alberta Cancer Board).

1. Hall MN, Raff M, Thomas G (2004) *Cell Growth: Control of Cell Size* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY).
2. Fingar DC, Blenis J (2004) Target of rapamycin (TOR): An integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. *Oncogene* 23:3151–3171.
3. Wullschleger S, Loewith R, Hall MN (2006) TOR signaling in growth and metabolism. *Cell* 124:471–484.
4. Prober DA, Edgar BA (2001) Growth regulation by oncogenes—new insights from model organisms. *Curr Opin Genet Dev* 11:19–26.
5. Mirth CK, Riddiford LM (2007) Size assessment and growth control: How adult size is determined in insects. *Bioessays* 29:344–355.
6. Oldham S, Montagne J, Radimerski T, Thomas G, Hafen E (2000) Genetic and biochemical characterization of *dTOR*, the *Drosophila* homolog of the target of rapamycin. *Genes Dev* 14:2689–2694.
7. Zhang H, Stallock JP, Ng JC, Reinhard C, Neufeld TP (2000) Regulation of cellular growth by the *Drosophila* target of rapamycin *dTOR*. *Genes Dev* 14:2712–2724.
8. Layalle S, Arquier N, Léopold P (2008) The TOR pathway couples nutrition and developmental timing in *Drosophila*. *Dev Cell* 15:568–577.
9. Géminard C, Rulifson EJ, Léopold P (2009) Remote control of insulin secretion by fat cells in *Drosophila*. *Cell Metab* 10:199–207.
10. Ma XM, Blenis J (2009) Molecular mechanisms of mTOR-mediated translational control. *Nat Rev Mol Cell Biol* 10:307–318.
11. Teleman AA, Chen YW, Cohen SM (2005) 4E-BP functions as a metabolic brake used under stress conditions but not during normal growth. *Genes Dev* 19:1844–1848.
12. Zaragoza D, Ghavidel A, Heitman J, Schultz MC (1998) Rapamycin induces the G0 program of transcriptional repression in yeast by interfering with the TOR signaling pathway. *Mol Cell Biol* 18:4463–4470.
13. Woiwode A, et al. (2008) PTEN represses RNA polymerase III-dependent transcription by targeting the TFIIIB complex. *Mol Cell Biol* 28:4204–4214.
14. White RJ (2008) RNA polymerases I and III, non-coding RNAs and cancer. *Trends Genet* 24:622–629.
15. Upadhya R, Lee J, Willis IM (2002) Maf1 is an essential mediator of diverse signals that repress RNA polymerase III transcription. *Mol Cell* 10:1489–1494.
16. Michels AA, et al. (2010) mTORC1 directly phosphorylates and regulates human MAF1. *Mol Cell Biol* 30:3749–3757.
17. Kantidakis T, Ramsbottom BA, Birch JL, Dowding SN, White RJ (2010) mTOR associates with TFIIIC, is found at tRNA and 5S rRNA genes, and targets their repressor Maf1. *Proc Natl Acad Sci USA* 107:11823–11828.
18. Marshall L, Kenneth NS, White RJ (2008) Elevated tRNA(iMet) synthesis can drive cell proliferation and oncogenic transformation. *Cell* 133:78–89.
19. Sonenberg N, Hinnebusch AG (2009) Regulation of translation initiation in eukaryotes: Mechanisms and biological targets. *Cell* 136:731–745.
20. Struhl G, Basler K (1993) Organizing activity of wingless protein in *Drosophila*. *Cell* 72:527–540.
21. Leopold P, Perrimon N (2007) *Drosophila* and the genetics of the internal milieu. *Nature* 450:186–188.
22. Delanoue R, Slaidina M, Léopold P (2010) The steroid hormone ecdysone controls systemic growth by repressing dMyc function in *Drosophila* fat cells. *Dev Cell* 18:1012–1021.
23. Brogiolo W, et al. (2001) An evolutionarily conserved function of the *Drosophila* insulin receptor and insulin-like peptides in growth control. *Curr Biol* 11:213–221.
24. Ikeya T, Galic M, Belawat P, Nairz K, Hafen E (2002) Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in *Drosophila*. *Curr Biol* 12:1293–1300.
25. Puig O, Marr MT, Ruhf ML, Tjian R (2003) Control of cell number by *Drosophila* FOXO: Downstream and feedback regulation of the insulin receptor pathway. *Genes Dev* 17:2006–2020.
26. Miron M, et al. (2001) The translational inhibitor 4E-BP is an effector of PI(3)K/Akt signalling and cell growth in *Drosophila*. *Nat Cell Biol* 3:596–601.
27. Lachance PE, Miron M, Raught B, Sonenberg N, Lasko P (2002) Phosphorylation of eukaryotic translation initiation factor 4E is critical for growth. *Mol Cell Biol* 22:1656–1663.
28. Gaal T, Bartlett MS, Ross W, Turnbough CL, Jr., Gourse RL (1997) Transcription regulation by initiating NTP concentration: rRNA synthesis in bacteria. *Science* 278:2092–2097.
29. Colombani J, et al. (2003) A nutrient sensor mechanism controls *Drosophila* growth. *Cell* 114:739–749.
30. Preiss T, Baron-Benhamou J, Ansorge W, Hentze MW (2003) Homodirectional changes in transcriptome composition and mRNA translation induced by rapamycin and heat shock. *Nat Struct Biol* 10:1039–1047.
31. Bilanges B, et al. (2007) Tuberous sclerosis complex proteins 1 and 2 control serum-dependent translation in a TOP-dependent and -independent manner. *Mol Cell Biol* 27:5746–5764.
32. Mamane Y, et al. (2004) eIF4E—from translation to transformation. *Oncogene* 23:3172–3179.
33. Zid BM, et al. (2009) 4E-BP extends lifespan upon dietary restriction by enhancing mitochondrial activity in *Drosophila*. *Cell* 139:149–160.
34. Rogers AN, et al. (2011) Life span extension via eIF4G inhibition is mediated by posttranscriptional remodeling of stress response gene expression in *C. elegans*. *Cell Metab* 14:55–66.
35. Colombani J, et al. (2005) Antagonistic actions of ecdysone and insulins determine final size in *Drosophila*. *Science* 310:667–670.
36. Caldwell PE, Walkiewicz M, Stern M (2005) Ras activity in the *Drosophila* prothoracic gland regulates body size and developmental rate via ecdysone release. *Curr Biol* 15:1785–1795.
37. Mirth C, Truman JW, Riddiford LM (2005) The role of the prothoracic gland in determining critical weight for metamorphosis in *Drosophila* melanogaster. *Curr Biol* 15:1796–1807.
38. Walkiewicz MA, Stern M (2009) Increased insulin/insulin growth factor signaling advances the onset of metamorphosis in *Drosophila*. *PLoS ONE* 4:e5072.
39. Silvera D, Formenti SC, Schneider RJ (2010) Translational control in cancer. *Nat Rev Cancer* 10:254–266.
40. Fontana L, Partridge L, Longo VD (2010) Extending healthy life span—from yeast to humans. *Science* 328:321–326.
41. Mehta R, Chandler-Brown D, Ramos FJ, Shamieh LS, Kaeberlein M (2010) Regulation of mRNA translation as a conserved mechanism of longevity control. *Adv Exp Med Biol* 694:14–29.