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Nutrient/TOR-dependent regulation of RNA polymerase III controls tissue and organismal growth in *Drosophila*

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

[1]Department of Biochemistry and Molecular Biology, Clark H Smith Brain Tumour Centre, Southern Alberta Cancer Research Institute, University of Calgary, HRIC, Calgary, Alberta, Canada

The nutrient/target-of-rapamycin (TOR) pathway has emerged as a key regulator of tissue and organismal growth in metazoans. The signalling components of the nutrient/TOR pathway are well defined; however, the downstream effectors are less understood. Here, we show that the control of RNA polymerase (Pol) III-dependent transcription is an essential target of TOR in *Drosophila*. We find that TOR activity controls Pol III in growing larvae via inhibition of the repressor Maf1 and, in part, via the transcription factor *Drosophila* Myc (dMyc). Moreover, we show that loss of the Pol III factor, Brf, leads to reduced tissue and organismal growth and prevents TOR-induced cellular growth. TOR activity in the larval fat body, a tissue equivalent to vertebrate fat or liver, couples nutrition to insulin release from the brain. Accordingly, we find that fat-specific loss of Brf phenocopies nutrient limitation and TOR inhibition, leading to decreased systemic insulin signalling and reduced organismal growth. Thus, stimulation of Pol III is a key downstream effector of TOR in the control of cellular and systemic growth.

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Introduction

An important question in developmental biology concerns the mechanisms that control growth and final size in multicellular animals. Studies in different model organisms have identified many conserved cell–cell secreted factors and signalling pathways that control growth. One key regulator that has emerged from this work is the serine/threonine kinase, target-of-rapamycin (TOR; for reviews, see De Virgilio and Loewith, 2006; Wullschleger *et al*, 2006 and Foster and Fingar, 2010).

*Corresponding author. Department of Biochemistry and Molecular Biology, Clark H Smith Brain Tumour Centre, Southern Alberta Cancer Research Institute, University of Calgary, HRIC, 3330 Hospital Drive, Calgary, Alberta, Canada T2N 4N1. Tel.: +1 403 210 6535; Fax: +1 403 210 9563; E-mail: grewalss@ucalgary.ca

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From yeast to mammals, TOR activity is cell-autonomously stimulated by an array of extracellular cues such as amino acids, glucose and oxygen to control growth and proliferation (Arsham and Neufeld, 2006; Dann and Thomas, 2006; Wullschleger *et al*, 2006; Hietakangas and Cohen, 2009; Wang and Proud, 2009; Foster and Fingar, 2010). In addition, in metazoans TOR can be activated by an endocrine insulin/insulin-like growth factor (IGF) signalling pathway (Oldham and Hafen, 2003; Grewal, 2008; Teleman, 2009). Insulins and insulin-like peptides bind to receptors on the surface of target cells. Ligand-receptor binding then triggers a conserved intracellular signalling cascade involving phosphoinositol 3-kinase (PI3K) and Akt, ultimately leading to increased TOR activity (Bhaskar and Hay, 2007; Efeyan and Sabatini, 2009). While these cell–cell and intracellular signalling inputs to TOR are well defined, the key downstream outputs by which TOR mediates its effects on metabolism and growth *in vivo* are less clear.

Considerable attention has focussed on the role of cellular protein synthesis as a regulator of cell growth. Extensive studies in mammalian cell culture have identified several mechanisms by which TOR can control mRNA translation (for reviews, see Proud, 2007; Ma and Blenis, 2009 and Sonenberg and Hinnebusch, 2009). For example, TOR can phosphorylate and inhibit the translational repressor eukaryotic initiation factor 4E-binding protein (4E-BP) leading to stimulation of protein synthesis (Thomas, 2002; Jastrzebski *et al*, 2007; Ma and Blenis, 2009). This translational mechanism is widely proposed as a key growth-regulatory target of TOR signalling (Dowling *et al*, 2010). These effects may not, however, account fully for the *in vivo* growth functions of TOR. For example, in *Drosophila,* TOR null mutants are lethal with severe growth defects (Oldham *et al*, 2000; Zhang *et al*, 2000) and overactivation of TOR signalling can promote considerable overgrowth; null mutants for 4E-BP, on the other hand, are viable with no effects on growth during development (Miron *et al*, 2001; Teleman *et al*, 2005). The regulation of ribosome synthesis is another TOR function important for protein synthesis and growth. Studies in yeast and mammalian cell culture have identified several mechanisms by which TOR can control the expression of ribosomal RNA (rRNA) and ribosome biogenesis genes (Mayer and Grummt, 2006). Moreover, recent work in *Drosophila* has emphasized the *in vivo* regulation of ribosome synthesis by TOR. For example, in larvae the insulin/TOR pathway controls the expression of ribosome synthesis genes via the transcription factors FOXO and Myc (Teleman *et al*, 2008; Li *et al*, 2010). In addition, the RNA polymerase I factor, TIF-IA, is required for rRNA synthesis and larval growth and is a downstream target of insulin/TOR signalling (Grewal *et al*, 2007).

In this paper, we explore the regulation of RNA polymerase (Pol) III-dependent transcription as a growth-regulatory output

of insulin/TOR signalling in *Drosophila*. Pol III is responsible for the synthesis of small non-coding RNAs that are essential for mRNA translation (e.g., 5S rRNA and transfer RNAs—tRNAs). Thus, control of Pol III may therefore represent another mechanism by which TOR alters protein synthesis to regulate growth. Studies on TOR signalling and Pol III have been exclusively limited to work in yeast and mammalian cell culture studies. For example, the multisubunit transcription factor TFIIIB is essential for Pol III transcription initiation, and nuclear extracts from either nutrient-deprived or TOR-inhibited yeast show reduced TFIIIB activity *in vitro* (Dieci *et al*, 1995; Sethy *et al*, 1995; Clarke *et al*, 1996; Zaragoza *et al*, 1998). Furthermore, in cultured mammalian cells the Brf (TFIIIB-related factor) subunit of TFIIIB is regulated downstream of several growth-regulatory signalling pathways including the TOR cascade (Goodfellow and White, 2007; Woiwode *et al*, 2008). These effects on TFIIIB/Pol III-dependent transcription in yeast and mammalian cells may reflect the ability of TOR to phosphorylate and inhibit the Pol III repressor Maf1, thus promoting transcription (Upadhya *et al*, 2002; Lee *et al*, 2009; Wei *et al*, 2009; Kantidakis *et al*, 2010; Michels *et al*, 2010; Shor *et al*, 2010). Mammalian Brf activity can also be stimulated by direct interaction with oncogenes such as c-Myc (White, 2005). While these *in vitro* studies have provided important molecular details about the regulation of Pol III *in vitro*, they do not address questions about metabolism, growth and size control in a developing multicellular animal: How does regulation of Pol III influence cell and tissue growth? Is Pol III required for the *in vivo* functions of TOR? If so, what are the regulatory mechanisms involved?

Our approach has been to use *Drosophila* as a model system to examine the contribution of Pol III-dependent transcription to the control of cell and tissue growth *in vivo*. During *Drosophila* larval development, the period of the life cycle characterized by an immense increase in growth, the major function of TOR signalling is to couple dietary nutrition to cell and tissue growth (Britton *et al*, 2002). TOR activity is required to cell-autonomously control growth in all larval tissues. In addition, stimulation of TOR in specific tissues can also play a non-autonomous role in systemic growth. For example, in well-fed larvae, amino-acid import into fat cells activates TOR leading to relay of a signal to the brain to promote the release of several *Drosophila* insulin-like peptides (dILPs) from discrete neurosecretory cells (Ikeya *et al*, 2002; Geminard *et al*, 2009). These dILPs then circulate through the larval haemolymph and activate the insulin-signalling pathway to stimulate cell growth in all larval tissues. We show here that Brf is an essential effector of TOR in the control of both cell-autonomous and non-autonomous effects on growth and body size in *Drosophila*. Moreover, we present evidence for a prominent role for dMaf1, but only a limited role for *Drosophila* Myc (dMyc), in the control of Pol III by nutrient-TOR signalling in developing animals.

Results

Brf is required for both cellular and organismal growth in Drosophila larvae

Brf, a conserved component of the TFIIIB complex, is limiting for Pol III-dependent transcription in yeast and mammals (Geiduschek and Kassavetis, 2001; Marshall *et al*, 2008). We therefore investigated if Brf is involved in controlling Pol III-dependent transcription and growth in *Drosophila* larvae. For these experiments, we analysed two publicly available lines (Bloomington Stock Center) carrying *P*-element insertions in the *brf* locus ($brf^{EY02964}$ and brf^{c07161}). Homozygous $brf^{EY02964}$ flies were lethal and this lethality could be rescued by ubiquitous *GAL4*-dependent expression of a *UAS-brf* transgene. Homozygous $brf^{EY02964}$ larvae also had reduced levels of both Brf protein (Figure 1A) and Pol III-dependent transcripts (Figure 1B) compared with control, wild-type larvae at the same developmental stage. Furthermore, levels of 7SL RNA were lower in *brf* mutants compared with controls; however, we did not detect any changes in the levels of 5S rRNA or the Pol I-dependent transcript, pre-rRNA (Supplementary Figure S1). Phenotypically, $brf^{EY02964}$ larvae progressed through embryogenesis but arrested as second instar larvae, surviving for several days (Figure 1C). A similar phenotype was seen in flies transheterozygous for $brf^{EY02964}$ and a deficiency that uncovers the *brf* locus (*Df(3R)BSC565*), suggesting that $brf^{EY02964}$ is either a null or strong hypomorphic loss-of-function allele of *brf*. We therefore used this line as a *brf* mutant. Flies that were homozygous for the second P-element line, brf^{c07161}, also exhibited lethality, but this could not be rescued by ubiquitous *GAL4*-dependent expression of a *UAS-brf* transgene. Hence, this P-element line must also be mutated in another essential gene, and so we did not study it any further. The growth inhibitory effects seen in homozygous *brf* mutant larvae could be phenocopied by expression of a *UAS-brf RNAi* construct using the ubiquitous *daughterless* (*da)-GAL4* driver (Supplementary Figure S2). Reducing Brf protein levels in this manner also decreased rates of Pol III-dependent transcription (Supplementary Figure S2A and B) and reduced larval growth rates (Supplementary Figure S2C). Expression of *brf RNAi* in either the salivary gland (*patched* (*ptc*); Supplementary Figure S2D and E) or eye imaginal discs (*eyeless* (*ey*); data not shown) also led to a reduction in tissue growth. Importantly, the growth inhibitory effects of the *brf RNAi* transgene were reversed by overexpression of *UAS-brf*, indicating that the RNAi-mediated effects were specifically due to Brf knockdown (Supplementary Figure S2F and G). In contrast to the effects of Brf inhibition, we found that overexpression of Brf alone was not sufficient to stimulate Pol III activity or affect organismal growth (data not shown). Thus, Brf, probably through its role in driving Pol III-dependent transcription, is essential for both tissue and organismal growth in *Drosophila* larvae.

We examined whether these growth defects observed following the loss of Brf function were due to inhibition of cellular growth. Most of the mass increase in developing larvae occurs in endoreplicating cells that make up the bulk of larval organs, such as the *Drosophila* fat body. Using mosaic analysis, we found that *brf* mutant cells (Figure 1D, GFP negative) in the larval fat body showed a marked decrease in size compared with surrounding heterozygote and wild-type cells (Figure 1D, GFP positive). We also created mosaic *brf* clones in the mitotically dividing cells of the larval wing imaginal disc. At 48 h following the clone induction, *brf* clones showed a growth defect and were approximately half the size of sister wild-type twin spots (Figure 1E). We also used flow cytometry to measure the size of dissociated wing

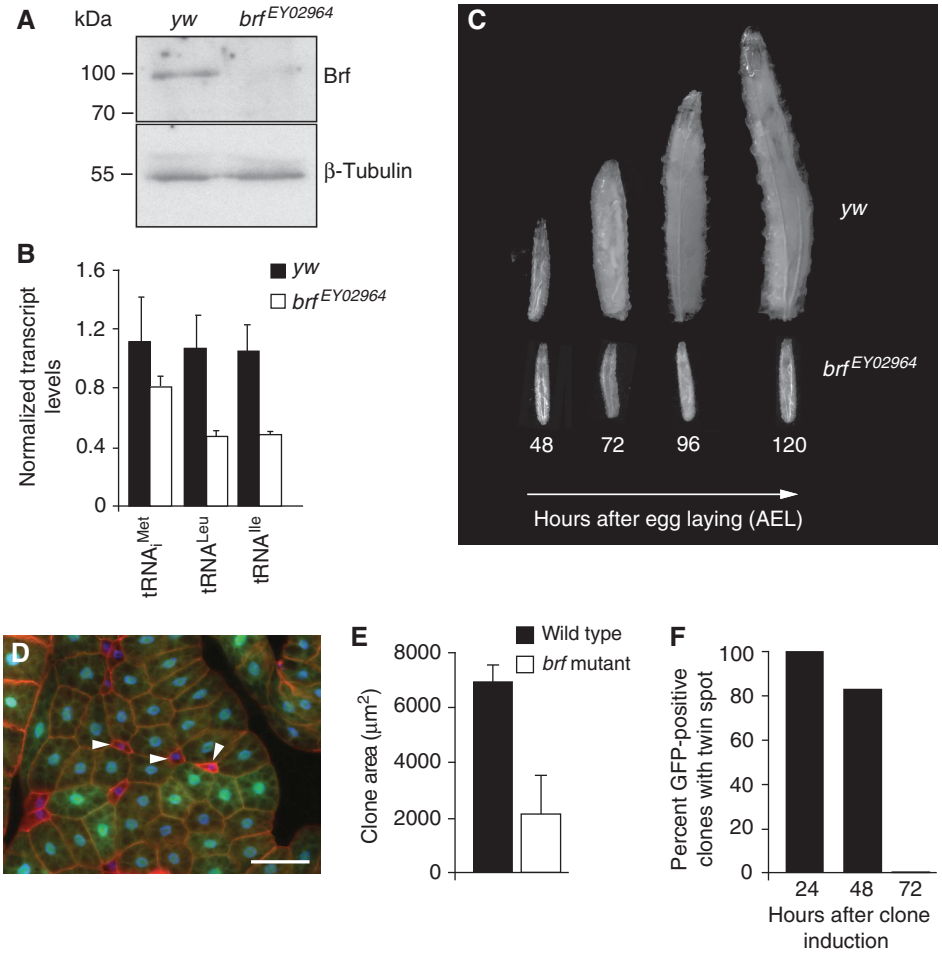

Figure 1 Loss of Brf function leads to severe growth defects in *Drosophila* larvae. (**A**) Brf protein levels were reduced in *brf* mutant ($brf^{EY02964}$) larvae compared with controls (*yw*) 48 h after egg laying (AEL), as determined by immunoblot. (**B**) Levels of Pol III-dependent transcripts were significantly decreased in *brf* mutant larvae compared with control larvae 48 h AEL, as measured by qRT–PCR ($P<0.05$, Student's *t*-test). Error bars indicate s.e.m. (**C**) *brf* mutant larvae are growth arrested. Images of control and *brf* mutant larvae throughout larval development (48–120 h AEL) are shown. (**D**) *brf* clones (non-GFP, arrowheads) were induced by *flp/FRT*-mediated recombination during embryogenesis and visualized in the fat body 120 h AEL. Blue, DAPI staining; red, actin; green, GFP. Scale bar, 100 μm. (**E**) *brf* mutant clones in wing discs were induced 72 h AEL and clone areas measured 120 h AEL ($n=100$ twin spots). (**F**) *brf* mutant clones were induced and scored in wing imaginal discs at the times indicated post clone induction. The viability of mutant clones was assessed by counting the percentage of wild-type clones that were still paired with a *brf* mutant twin spot. Genotypes used in (**D**–**F**): $hsflp^{122}$; +; *FRT82B,* $brf^{EY02964}$*/FRT82B, ubi-GFP.*

disc cells at this time point, and found that *brf* cells showed little change in size compared with wild-type and *brf* heterozygous cells (data not shown). Together, these data suggest that loss of Brf leads to a coordinated decrease in both cellular growth (mass increase) and cell division in the wing disc, resulting in fewer, slower dividing cells that maintain a normal size. We also found that the viability of the *brf* mutant clones decreased with time following the clone induction. Approximately 80% of wild-type clones still had sister *brf* mutant twin-spot clone at 48 h after clone induction; however, at 72 h post induction none of the wild-type clones we examined still had a *brf* mutant sister clone (Figure 1F). This result suggests that slower growing and dividing *brf* mutant cells are outcompeted and eliminated by their faster growing neighbours. Consistent with this interpretation, we could rescue the viability of *brf* mutant cells by genetically reducing the growth rate of surrounding cells by making them heterozygous for a dominant *Minute (M)* allele of ribosomal protein S3. In this case, we could recover *brf* mutant clones at 72 h after clone induction, a time point at which all clones are normally eliminated (Supplementary Figure S3A and B). These rescued *brf* clones still showed a strong growth defect compared with wild-type cells. Moreover, we found that overexpression of the baculovirus anti-apoptotic protein p35 in the *brf* mutant wing disc cells rescued clone viability at 72 h post clone induction. These rescued *brf* mutant clones were, however, still smaller than wild-type clones (Supplementary Figure S3C–E; clones of each genotype are GFP positive). Together, these data show that Brf function is required for Pol III-dependent transcription and cell and organismal growth during *Drosophila* development.

Brf activity in fat cells is required to maintain systemic insulin signalling and organismal growth

The *brf* mutant phenotype is reminiscent of the phenotype of larvae raised in nutritionally poor conditions. In *Drosophila* larvae, the function of nutrient sensing is performed by the

fat body, an insect organ that performs both endocrine and nutrient storage functions similarly to the vertebrate liver and adipose tissue. In nutrient-rich conditions, the fat body signals to the brain to promote release of several dILPs, thereby promoting systemic insulin signalling and growth. We therefore explored whether changes in Pol III activity play a role in the *Drosophila* fat body in controlling organismal growth in a cell non-autonomous manner. To address this, we expressed a *brf RNAi* transgene specifically in the *Drosophila* fat body and examined the effects on the growth and development of the larvae. We found that silencing *brf* in the fat body (*r4>brf RNAi*) reduced larval growth rates and delayed pupation, with ~15% of larvae failing to pupate and remaining as third instar larvae (Figure 2A). At wandering third instar stage, *r4>brf RNAi* larvae had significantly smaller wing imaginal discs than control larvae (Figure 2B). Subsequently, we found that *r4>brf RNAi* adults were smaller than controls and weighed less (Figure 2C). Similar but stronger effects were seen using another fat body driver (*cg-GAL4*); *cg>brf RNAi* larvae were significantly smaller than controls and failed to progress into the pupal stage (Supplementary Figure S4A).

We explored whether these organismal growth effects caused by inhibiting Brf in the fat body were a consequence of reduced systemic insulin signalling. To do so, we first examined phospho-Akt levels in the peripheral tissues of *r4>brf RNAi* animals by immunoblotting. Akt is a key downstream effector of the insulin pathway, and Akt activity can be measured by assaying for phosphorylation of a carboxy terminus serine residue at position 505. We found that phosphorylation of Akt at serine 505 was reduced in *r4>brf RNAi* larvae peripheral tissues (larval carcasses devoid of fat body), even though total Akt was still present at levels comparable to control animals (Figure 2D). Similarly,

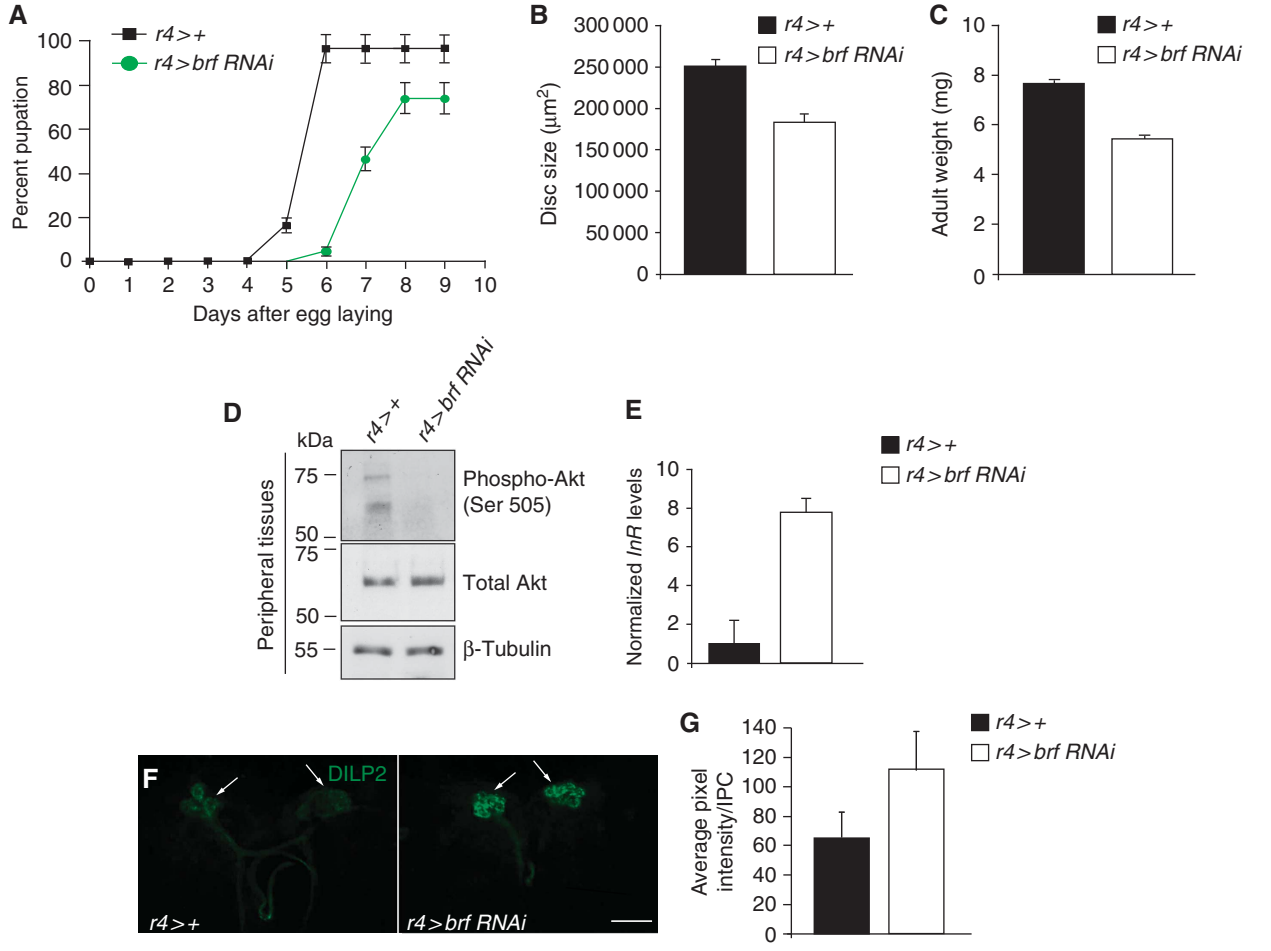

Figure 2 Fat body-specific reduction in Brf activity has cell non-autonomous effects on organismal growth and development. (**A**) Fat body-specific reduction in Brf levels (*r4>brf RNAi*, green trace) delayed pupariation when compared with controls (*r4>+*, black trace). The data are represented as a percentage of larvae that develop to pupal stage. Error bars indicate s.e.m. (**B**) Fat body loss of Brf function results in smaller wing imaginal discs, compared with controls ($n>20$ per genotype). Disc size was quantified in wandering third instar larvae using the histogram tool (Adobe Photoshop). Error bars indicate standard error. (**C**) Reduced Brf in the fat body reduced adult weight compared with control adults. Error bars indicate standard error. (**D**–**G**) Silencing of *brf* specifically in the *Drosophila* fat body decreased peripheral insulin signalling. (**D**) *brf* silencing in the fat body abolished Akt phosphorylation at serine 505 in peripheral tissues while total Akt protein levels remained constant. Levels of β-tubulin were measured to ensure equal loading. (**E**) Decreasing Brf levels specifically in the fat body using the *r4-GAL4* driver significantly increased *dInR* mRNA levels in peripheral tissues of these animals when compared with controls ($P<0.05$, Student's *t*-test; $n=32$ per genotype). All qRT–PCR error bars represent s.e.m. (**F**) There is an accumulation of dILP2 protein in the IPCs of *r4>brf RNAi* larval brains compared with controls (*r4>+*). (**G**) Quantified pixel intensities of DILP2 staining in IPC clusters in the larval brain (*r4>+*, $n=12$ and *r4>brf RNAi*, $n=21$; error bars represent standard deviation; $P=000174$). For (**D**–**G**), larvae were analysed at 96 h AEL.

brf mutant larvae also had lower levels of Akt phosphorylation at serine 505 compared with age-matched wild-type whole larvae (Supplementary Figure S4B). To further confirm that the inhibition of larval growth caused by fat body silencing of Brf was due to reduced systemic insulin signalling, we measured the levels of *dInR* mRNA. Transcription of this gene is negatively regulated by the insulin/PI3K pathway through the activation of FOXO (Puig and Tjian, 2005), and hence levels of *dInR* mRNA act as an additional readout of insulin signalling (Delanoue *et al*, 2010). When we used *r4-gal4* to drive *brf RNAi* in the fat body, we found an increase in *dInR* mRNA levels in peripheral tissues, consistent with a suppression of peripheral insulin signalling (Figure 2E). We saw a similar increase in *dInR* mRNA in both peripheral tissues from *cg>brf RNAi* (Supplementary Figure S4C), and also in *brf* mutant animals when compared with control animals (Supplementary Figure S4D). Finally, we examined whether these changes in systemic insulin signalling following the knockdown of Brf might be explained by either reduced expression or release of brain dILPs. Previous reports have shown that mRNA levels of *dilp5*, but not *dilp2*, are suppressed upon amino-acid starvation (Geminard *et al*, 2009). We saw no change in dILP mRNA expression levels in r4>*brf RNAi* larvae (Supplementary Figure S4E). In contrast, we saw reduced expression levels of the mRNAs encoding *dilp2* and *dilp5* in *cg>brf RNAi* larvae (Supplementary Figure S4F). We also found that *dilp5* mRNA levels were reduced in *brf* mutants (Supplementary Figure S4G). Amino-acid deprivation also leads to reduced release of dILPs from the brain and hence dILP proteins are retained in the neurosecretory insulin producing cells (IPCs) of starved animals (Geminard *et al*, 2009). Using immunostaining, we also found that dILP2 protein was retained in the IPCs of brains from *r4>brf RNAi* larvae compared with controls (Figure 2F and G). Taken together, these data suggest that Brf function and hence Pol III-dependent transcription is required in the fat body to maintain normal systemic insulin signalling and growth.

Given the organismal effects we observed following *brf* knockdown in fat cells, we examined whether Brf might be required for nutrient-dependent effects on fat body metabolism. To do so, we compared the fat bodies of starved larvae with those from larvae in which Brf had been specifically silenced in the fat body by expression of *brf RNAi* using the fat body driver *r4-GAL4*. Nutrient-deprivation/TOR inhibition induces marked changes in lipid metabolism (Colombani *et al*, 2003), which can be observed as an increase in lipid droplet size. Using both Differential Interference Contrast (DIC) microscopy and Nile Red staining, we observed an increased lipid droplet size in *r4>brf RNAi* larvae compared with control animals (Figure 3A, D versus C, F). These effects were similar to changes in lipid droplets in fat bodies dissected from either amino acid-deprived (Figure 3B and E) or *tor* mutant larvae (Zhang *et al*, 2000). Similar effects were seen when we expressed the *UAS-brf RNAi* transgene with another fat body driver, *cg-GAL4* (Supplementary Figure S5A and B). Starvation for amino acids also stimulates a rapid induction of autophagy, a response that is required for organismal survival. We found that fat bodies from 4 h starved larvae showed a marked increase in autophagasomes by using lysotracker staining (Figure 3H). In contrast, we found that *r4>brf RNAi* fat bodies showed no induction of autophagy (Figure 3I), similarly to fat bodies dissected from fed larvae (Figure 3G). These results suggest that Brf and Pol III-dependent transcription in the *Drosophila* fat body are required for some but not all of the metabolic effects of nutrient availability.

Pol III transcription is stimulated by the TOR pathway

The cell and organismal changes in metabolism, physiology and growth that we described for loss of Brf function are similar to those seen following the inhibition of TOR signalling. We therefore explored whether TOR regulates Pol III-dependent transcription in *Drosophila*, and whether this regulation is required for the *in vivo* functions of TOR signalling. To address this, we first measured levels of Pol III-dependent transcripts, by qRT–PCR, in *Drosophila* larvae following the modulation of the TOR pathway. Starvation for dietary protein leads to inhibition of TOR activity in larvae (Oldham *et al*, 2000; Zhang *et al*, 2000). We found that larvae starved in 20% sucrose/PBS had reduced levels of several Pol III-dependent transcripts such as the tRNAs, 5S rRNA and 7SL RNA (Figure 4A). To further investigate the involvement of the TOR pathway in Pol III regulation *in vivo,* we performed gene expression analyses in larvae in which we genetically manipulated TOR signalling. We first found that *tor* null mutants had significantly reduced levels of Pol III-dependent transcripts compared with age-matched control larvae, (Figure 4B). We also found that cultured *Drosophila* S2 cells treated with the TOR-specific inhibitor, rapamycin, also had reduced levels of Pol III-dependent products (Supplementary Figure S6). Similarly, we found that overexpression of negative regulators of the TOR pathway, TSC1/2 using the *UAS-GAL4* system, resulted in a substantial reduction in Pol III-dependent transcript levels compared with control larvae (Figure 4C). Finally, we found levels of Pol III-dependent transcripts were reduced in homozygous mutants for S6 kinase, a key TOR effector (Figure 4D). We next asked if overactivation of the TOR pathway can increase Pol III-dependent transcription. To address this, we first ubiquitously expressed a *tsc1 RNAi* transgene using the *da-GAL4* driver and found that these larvae had significantly increased levels of each of the Pol III-dependent transcripts measured (Figure 4E). We then examined larvae expressing a constitutively active form of the downstream TOR effector S6K, and found that levels of Pol III-dependent transcripts were significantly elevated in these larvae compared with controls (Figure 4F). Taken together, these data demonstrate that the TOR pathway is necessary and sufficient to stimulate Pol III-dependent transcription in developing larvae, in part through activation of S6 kinase.

Brf is required for TOR-induced cell growth

Given that Pol III-dependent transcription is regulated by the insulin/TOR pathway *in vivo* in *Drosophila* larvae, we next wanted to examine whether Brf functions downstream of TOR in the control of growth. TSC1 and TSC2 function together as negative regulators of TOR signalling (Ito and Rubin, 1999; Gao and Pan, 2001; Potter *et al*, 2001; Tapon *et al*, 2001). As a consequence, loss of TSC1 or TSC2 function leads to a TOR-dependent increase in cellular growth in larval tissues. We examined whether the overgrowth induced by the loss of TSC1 function was dependent on Brf. We used *flp/FRT*-mediated recombination to generate mutant clones in the

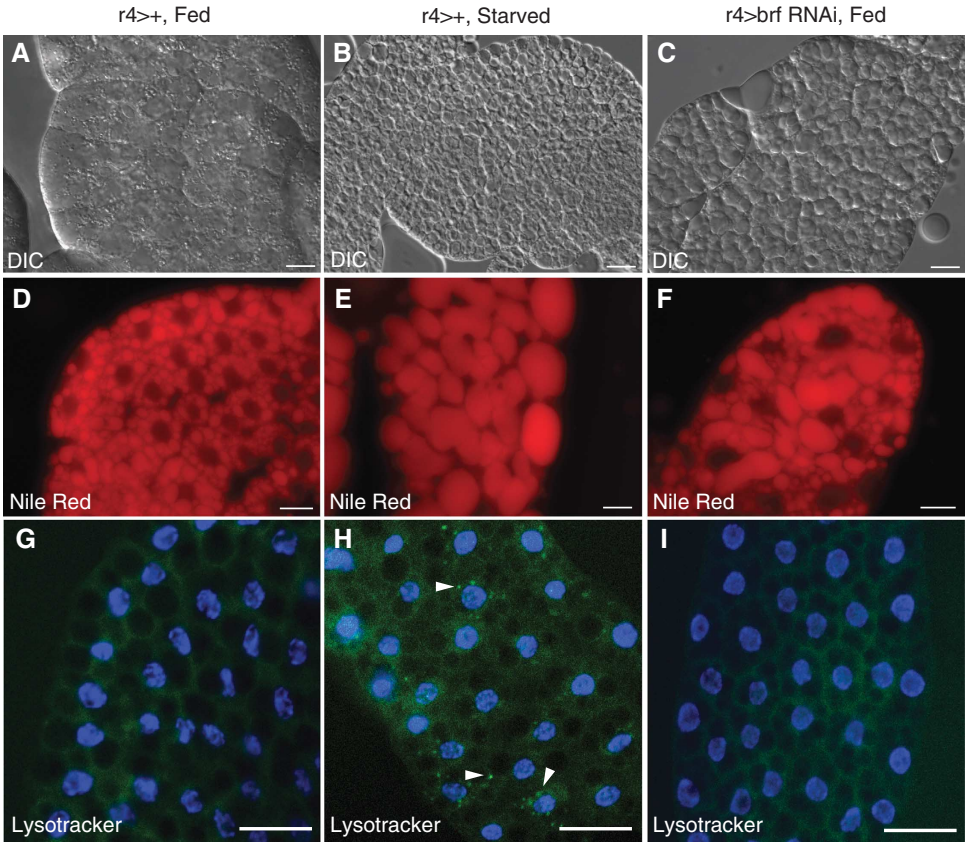

Figure 3 The fat body-specific loss of Brf function phenocopies some aspects of the starvation response. Fat bodies were dissected from 72 h larvae and stained with Nile Red to visualize lipid droplets or lysotracker green to vizualize autophagosomes. (**A**–**C**) DIC and (**D**–**F**) Nile Red images of fat bodies isolated from control (*r4>+*) fed (**A**, **D**) and control 24 h starved larvae (**B**, **E**). (**C**, **F**) Fed larvae with fat body-specific reduction in Brf levels (*r4>brf RNAi*) are shown. (**G**–**I**) Lysotracker green images of fat bodies isolated from control fed (**G**) and *r4>brf RNAi* (**I**) larvae show no lysotracker staining. Starved control larvae exhibit a punctuate staining pattern (**H**, arrowheads) caused by the formation of autophagosomes. Images were all taken at the same exposure. Scale bars, 100 μm.

wing imaginal discs (Figure 5A–C). As described above, *brf* mutant clones were growth defective and as a consequence were smaller than their wild-type twin spots (Figure 5A). In contrast, *tsc1* mutant clones were significantly larger than their wild-type twin spot, consistent with the growth promoting effects of increased TOR signalling (Figure 5B). However, we found that *brf, tsc1* double mutant clones were similar in size to the *brf* mutant clones (Figure 5C). The small clone size of either *brf* mutants or *brf, tsc1* double mutants could not be rescued by the expression of P35. Therefore, the small clone sizes were not merely a direct consequence of apoptosis (Supplementary Figure S7A–F). We performed similar mosaic clonal experiments in the larval fat body (Figure 5D–G). As in the wing discs, we found that *brf* mutant cells were smaller than controls (Figure 5D) and that *brf* was epistatic to *tsc1* (Figure 5F). Thus, *tsc1* cells exhibited a growth increase compared with surrounding cells (Figure 5E) while *brf, tsc1* double mutant cells phenocopied *brf* cells, and were severely growth impaired (Figure 5F and G). These data suggest that Brf is required for TOR-induced growth in both endoreplicating and mitotically dividing and cells of the larvae.

Drosophila Maf1 is the predominant regulatory link between nutrition/TOR and Pol III activity

We next wanted to address the mechanism by which TOR regulates Pol III-dependent transcription. We found that inactivation of TOR, by starving larvae for dietary protein, did not affect Brf protein levels (Supplementary Figure S8). One candidate we considered to be involved in controlling Pol III-dependent transcription downstream of TOR activity was the transcriptional repressor *Drosophila Maf1* (*dMaf1*, *CG40196*). In both yeast and mammalian cells, Maf1 represses Pol III activity and this repression, in turn, is reversed by nutrient/TOR signalling. We therefore examined if this mode of controlling Pol III was conserved in *Drosophila*. In feeding larvae, when insulin/TOR signalling is high, we found that ubiquitous expression of a *dMaf1 RNAi* transgene using the *da-GAL4* driver led to elevated levels of tRNAs compared with control larvae consistent with a role for dMaf1 as a repressor (Figure 6A–C). Similar effects on Pol III-dependent transcription were seen using another *dMaf1 RNAi* transgene that targets an overlapping but smaller region of *dMaf1* (Supplementary Figure S9A–C). Furthermore, the elevated tRNA levels seen in *da>Maf1 RNAi* larvae could be restored to wild-type levels by the expression of a *UAS-dMaf1* transgene (Supplementary Figure S9D and E), allowing us to conclude that these *dMaf-RNAi* effects on Pol III-dependent transcription were specific to loss of dMaf1. We found that inhibition of dMaf1 had no effect on transcript levels of components of the Pol III machinery, such as Brf, Trf1 or RPIII128 (Supplementary Figure S9F). We also found that inhibition of dMaf1 had no effect on levels of both pre-rRNA

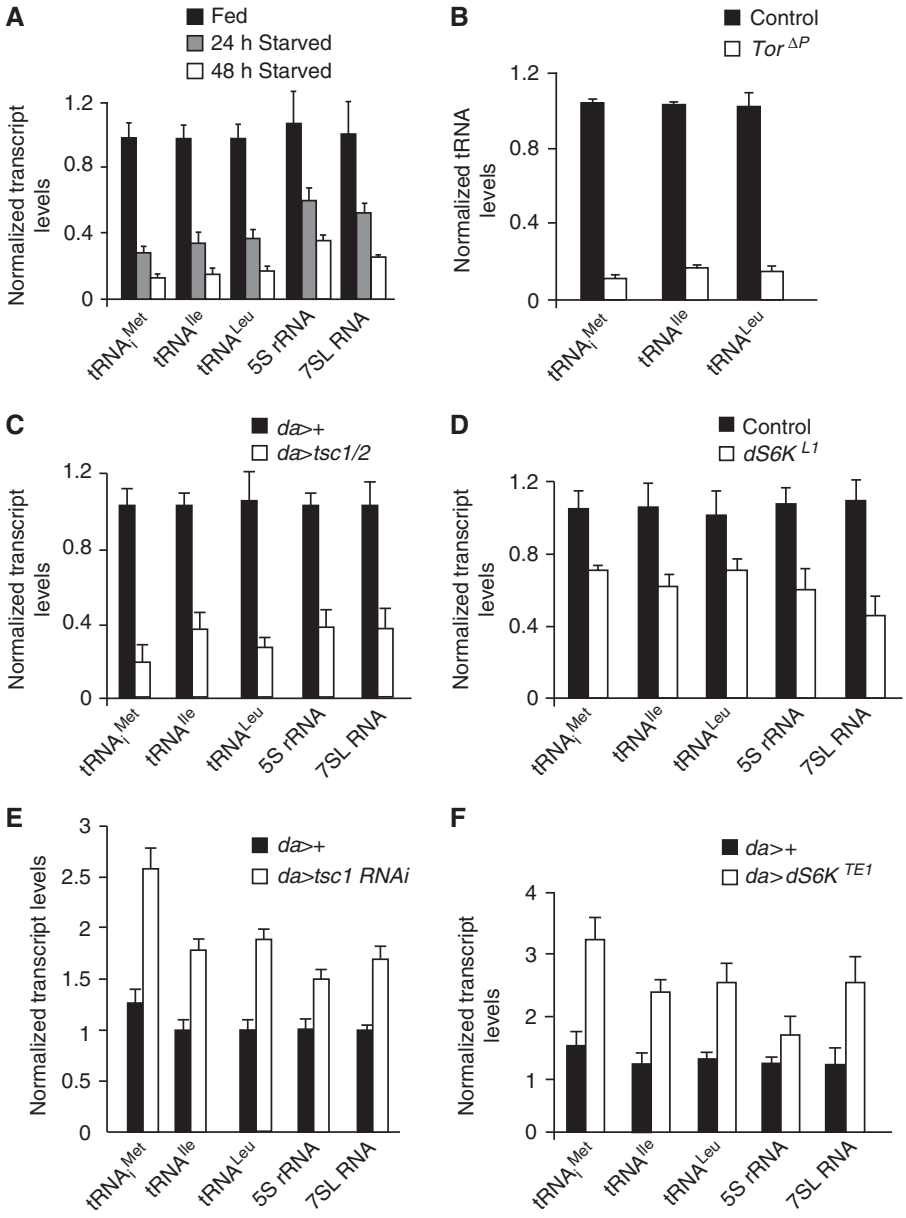

Figure 4 TOR signalling regulates Pol III-dependent transcription in *Drosophila* larvae. (**A**) Pol III-dependent transcripts were significantly decreased in wild-type (*yw*) larvae starved for dietary protein for 24 or 48 h compared with wild-type fed larvae. (**B**) tRNA levels were significantly decreased in $tor^{\Delta P}$ homozygous mutant larvae when compared with control (*yw*) larvae 48 h AEL ($P<0.05$, Student's *t*-test). (**C**) Pol III-dependent transcript levels were significantly decreased in larvae ubiquitously overexpressing *tsc1/2* (*da>tsc1/2*) compared with controls (*da>+*) larvae 48 h AEL ($P<0.05$, Student's *t*-test). (**D**) Levels of Pol III-dependent transcripts were significantly reduced in *S6K* homozygous ($dS6K^{L1}$) mutant larvae when compared with control (*yw*) larvae 48 h AEL ($P<0.05$, Student's *t*-test). (**E**) Levels of Pol III-dependent transcripts were elevated in whole larvae following the ubiquitous expression of a *tsc1 RNAi* transgene by *da-GAL4 (da>tsc1 RNAi)* compared with controls (*da>+*, $P<0.05$, Student's *t*-test) at 72 h AEL. (**F**) Ubiquitous expression of a constitutively active form of *S6K* ($da>S6K^{TE1}$) significantly increased Pol III-dependent transcript levels in whole larvae compared with control (*da>+*) larvae 72 h AEL ($P<0.05$, Student's *t*-test). Each experiment was independently performed three times with $n=32$ per genotype. For each qRT–PCR error bars indicate s.e.m.

and Ribosomal protein 49 mRNA. Thus, in contrast to a previous report on human Maf1 (Johnson *et al*, 2007), we find that *Drosophila* Maf1 has no effect on either Pol I- or Pol II-dependent transcription, and is probably a specific regulator of Pol III. As described above, starvation for dietary amino acids leads to reduced insulin/TOR signalling and consequently tRNA synthesis was suppressed (Figure 6A–C, compare fed versus starved in *da>+* animals). In starved *da>dMaf1 RNAi* larvae, however, we found that tRNA levels remained elevated (Figure 6A–C). Similar effects were seen when we used rapamycin feeding, instead of starvation, as a more specific way to inhibit TOR signalling (Figure 6D–F). Finally, we also found that activation of TOR signalling (by expression of a *tsc1 RNAi* transgene) only modestly augmented the effects of *dMaf1 RNAi* on tRNA levels (Supplementary Figure S9F). Together, these data argue that, in *Drosophila*, nutrient/TOR signalling stimulates Pol III and tRNA synthesis via inhibition of dMaf1. Maf1 is thought to function as a repressor by interacting with Brf1 and/or Pol III, sequestering these factors away from tRNA gene promoters. Using

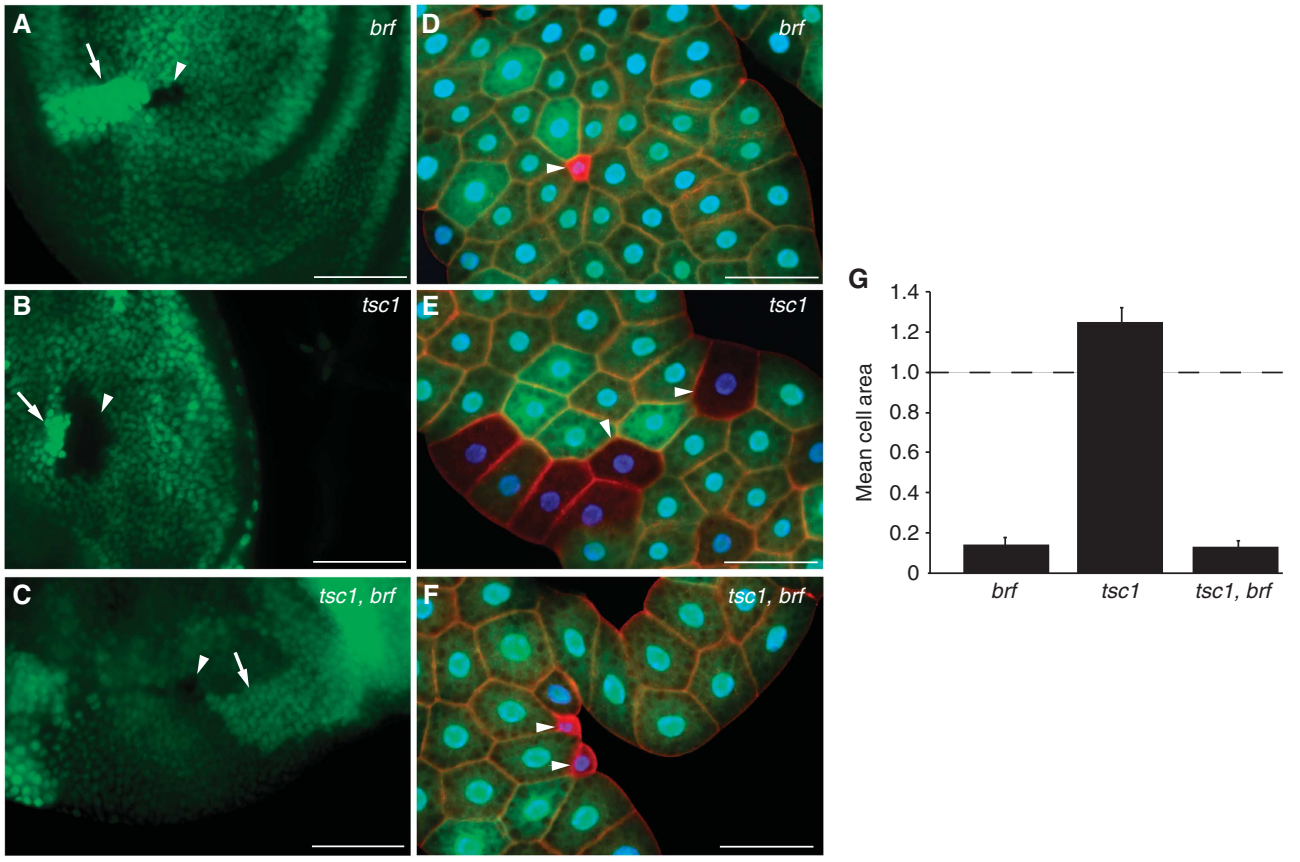

Figure 5 Brf is required for TOR-induced cell growth in both mitotically dividing and endoreplicating tissue in *Drosophila* larvae. (**A**–**F**) *brf*, *tsc1* or *tsc1,brf* double mutant clones were induced in both wing discs (**A**–**C**) and fat body (**D**–**G**). Mutant clones, arrowheads; wild-type sister clones, arrows. Blue, DAPI staining; red, actin; green, GFP. (**G**) The areas of both mutant and wild-type cells in the fat body were measured and presented here as mean cell area compared with control. Genotypes: (**A**, **D**) $hsflp^{122}$; +; *FRT82B*, $brf^{EY02964}$/*FRT82B*, *ubi-GFP*; (**B**, **E**) $hsflp^{122}$; +; *FRT82B*, $tsc1^{Q87X}$/*FRT82B*, *ubi-GFP*; (**C**, **F**) $hsflp^{122}$; +; *FRT82B*, $brf^{EY02964}$, $tsc1^{Q87X}$/*FRT82B*, *ubi-GFP*. (**A**–**C**) Scale bar, 50 μm. (**D**–**F**) Scale bar, 100 μm.

Drosophila cultured S2 cells, we found under normal conditions, when TOR activity was high, dMaf1 and Brf showed a weak association as measured by co-immunoprecipitation (Figure 6G). This interaction was, however, enhanced following the inhibition of TOR by rapamycin treatment (Figure 6G). Therefore, one mechanism by which dMaf1 may repress Pol III-dependent transcription under low TOR activity is to bind and sequester Brf away from Pol III promoters. Together, these data suggest that during larval development nutrient-dependent insulin/TOR signalling stimulates Pol III-dependent transcription through the inhibition of dMaf1.

dMyc activates Pol III transcription in vivo by two distinct mechanisms, but is not the major mediator of nutrition/TOR signalling

In both mammalian and *Drosophila* cultured cells, Myc can interact with Brf and stimulate Pol III-dependent transcription (Gomez-Roman *et al*, 2003; Steiger *et al*, 2008). We therefore explored the involvement of dMyc in regulating Pol III-dependent transcription *in vivo*. First, we examined *dMyc* null mutants and found that both tRNA levels and mRNA levels of components of the Pol III machinery—*Brf*, Tata Binding Protein (TBP)-related factor (*Trf*) and *RNA polymerase III subunit 128* (*RpIII128*)—were lower than in control larvae (Figure 7A). Conversely, we found that when we overexpressed a *UAS-dMyc* transgene using the *flp-out* technique we observed significantly higher levels of tRNA and *Brf*, *Trf* and *RpIII128* mRNAs (Figure 7B). We also preformed immunoblot analysis of larval extracts and found an increase in Brf protein levels following *dMyc* overexpression (Figure 7C). Finally, we performed a co-immunoprecipitation experiment in larval extracts, and using antisera directed against Brf, we identified an association between dMyc and Brf (Figure 7D). In contrast, mouse or rabbit immunoglobulins or antisera against TBP, which does not participate in initiating Pol III-dependent transcription in *Drosophila* (Takada *et al*, 2000), did not pull down dMyc (Figure 7D). Together, these data suggest that during *Drosophila* development, dMyc can promote Pol III-dependent transcription by at least two mechanisms: by controlling the levels of components of the Pol III apparatus and by directly associating with Brf. We explored whether these effects on Pol III were required for dMyc-induced growth. Previous studies have shown that, like TOR activity, overexpression of dMyc in the larval fat body can promote systemic growth and hence increase body size (Delanoue *et al*, 2010). We found that the increased pupal volume seen following the fat body expression of dMyc (*cg>dMyc*) was suppressed by co-expression of *brf RNAi* (Figure 7E). Given that dMyc levels are also controlled by nutrient/TOR signalling (Teleman *et al*, 2008), dMyc may also be a possible

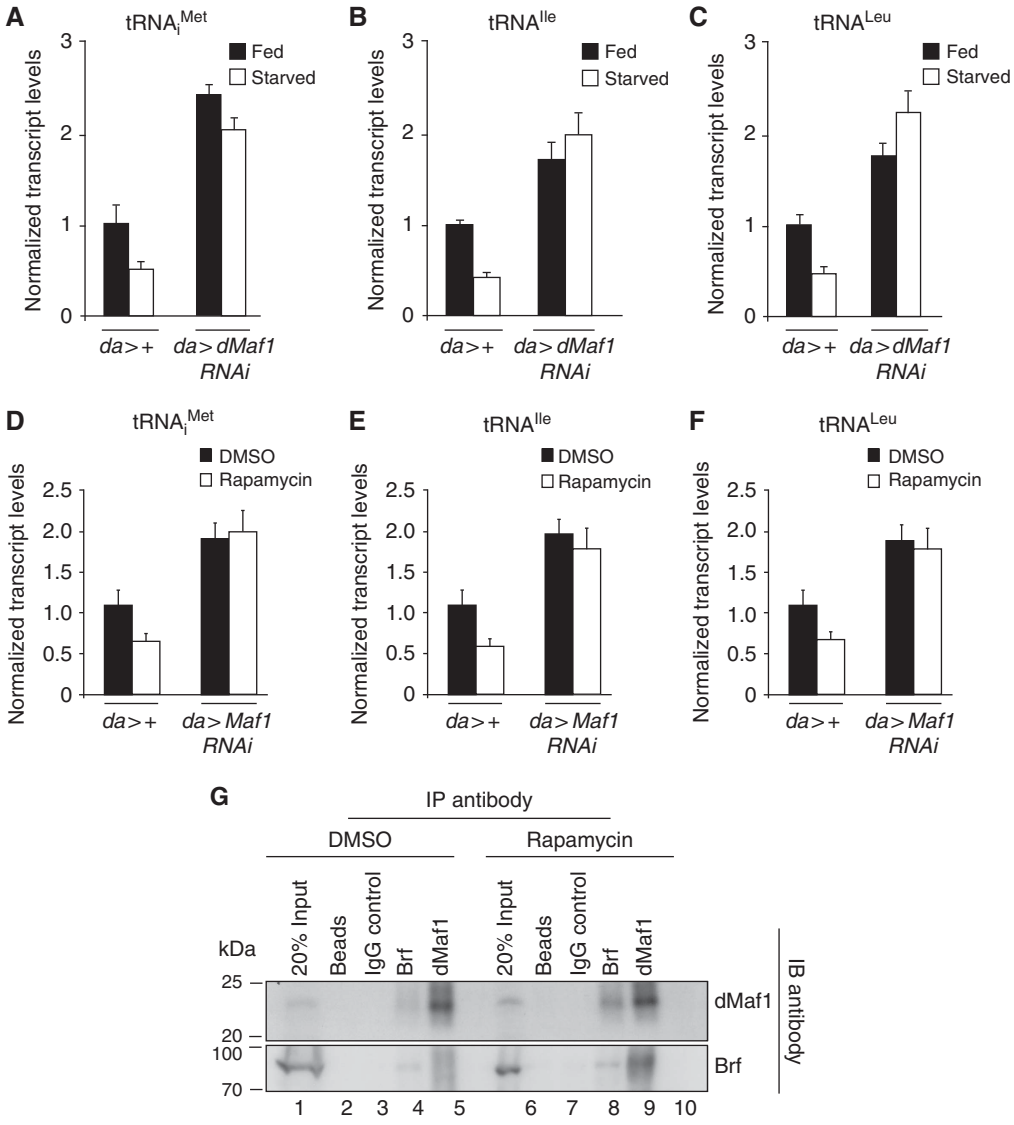

Figure 6 *Drosophila* Maf1 is the regulatory link between TOR and Pol III activity. (**A**–**F**) qRT–PCR analyses of RNA extracted from whole larvae following the ubiquitous expression of a *dMaf1 RNAi* transgene (*da>Maf1 RNAi*) compared with control (*da>+*) larvae. TOR activity was modulated either by starving the larvae of dietary protein (**A**–**C**) or by feeding larvae rapamycin (**D**–**F**). (**A**–**F**) tRNA levels were significantly elevated following loss of *dMaf1* (*da>Maf1 RNAi*) compared with controls (*da>+*) when TOR activity was high under normal fed or DMSO-treated conditions ($P<0.05$, Student's *t*-test). tRNA levels remain elevated in *da>Maf1 RNAi* animals even under starved or rapamycin-treated conditions when control tRNA levels are normally reduced ($P<0.05$, Student's *t*-test). For qRT–PCR error bars indicate s.e.m. (**G**) Immunoprecipitation of Brf and dMaf1 from *Drosophila* cultured S2 cell extracts revealed an enhanced association between Brf and dMaf1 following rapamycin treatment (compare lane 4 with 9 and lane 5 with 10).

candidate for the regulatory link between TOR and Brf. We found that the reduced tRNA levels seen in *dMyc* null mutants were not decreased further upon rapamycin treatment, suggesting that both TOR and dMyc may function in a linear pathway to control Pol III-dependent transcription (Figure 7F). To further investigate this, we asked whether maintaining high dMyc activity in *Drosophila* larvae could bypass the starvation induced decrease in tRNA synthesis. Our approach was to drive a *UAS-dMyc* transgene expression using the *flp-out* system and measure tRNA levels in both fed and starved larvae. As expected, we found that starvation led to a decrease in tRNA levels in control larvae (Figure 7G). Overexpression of *dMyc* stimulated tRNA synthesis in fed larvae, but only produced a modest increase in tRNA levels in starved animals (Figure 7G). This was not due to the fact that dMyc was less active in starved animals, since *PPAN*, a dMyc target gene, was as strongly induced in starved animals as in fed animals (Supplementary Figure S10A). Similar results on both tRNA and *PPAN* mRNA levels were obtained when we specifically inhibited TOR by rapamycin treating larvae overexpressing dMyc (Figure 7H; Supplementary Figure S10B). These findings with dMyc contrast with our findings with dMaf1 suppression, which was sufficient to completely bypass either the starvation- or rapamycin-induced inhibition of tRNA synthesis (see Figure 6). Together, our data suggest a model for Pol III regulation in *Drosophila* that is outlined in Figure 8.

Discussion

The TOR kinase is one of the best-established growth regulators (Wullschleger *et al*, 2006). In virtually all animals,

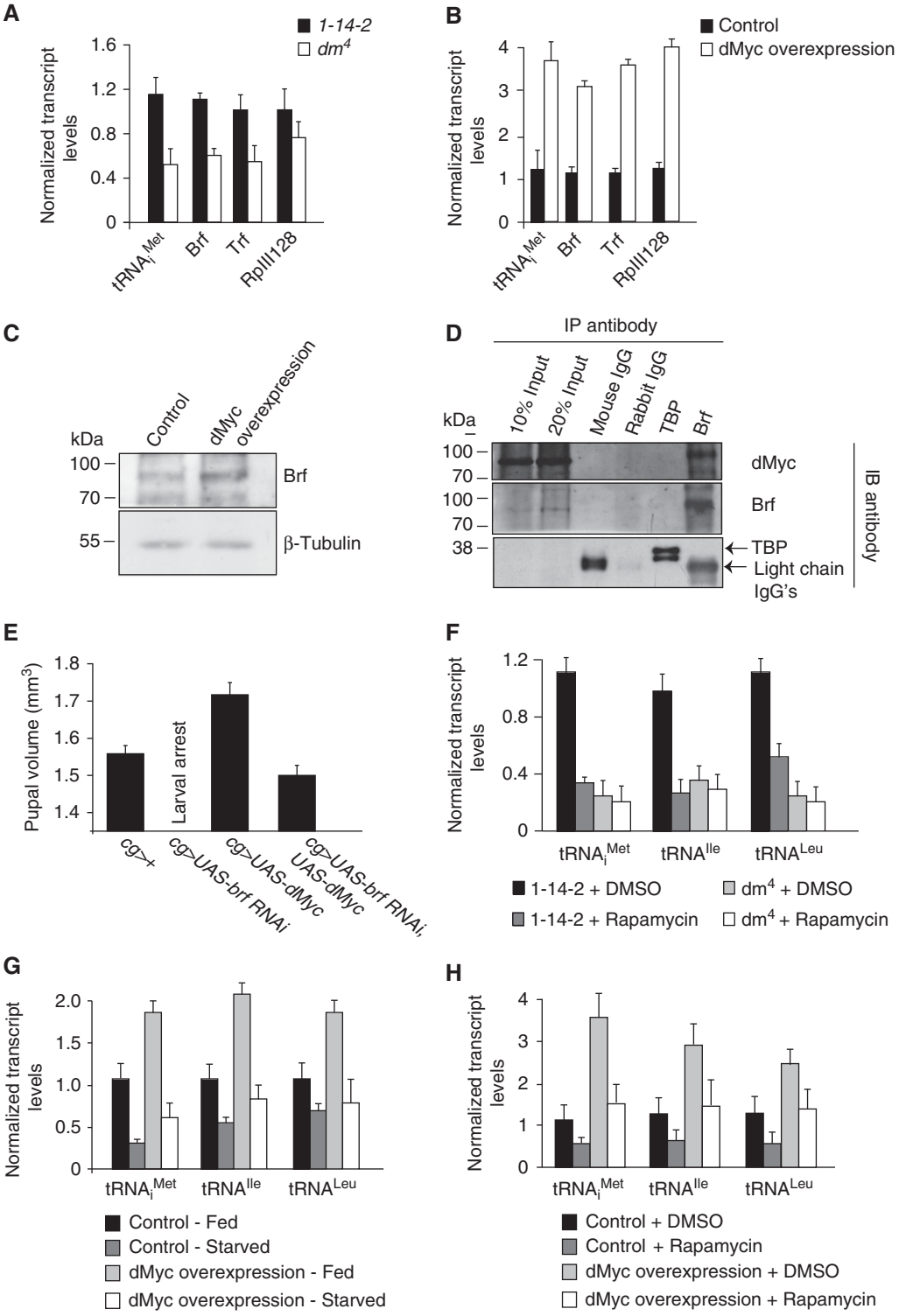

Figure 7 dMyc activates Pol III-dependent transcription *in vivo* by two distinct mechanisms. (**A**) $tRNA_i^{Met}$, *Brf* and *Trf* mRNA levels were significantly decreased in *dMyc* homozygous mutants (*dm*^{4}) compared with controls (*1-14-2*, $P<0.05$; Student's *t*-test) 48 h AEL. (**B**) tRNA synthesis and TFIIIB mRNA levels were significantly elevated in larvae following dMyc overexpression using the flp-out technique (*hsflp/+; +;UAS-dMyc/act>CD2>Gal4*) compared with controls (*hsflp/+;+;act>CD2>Gal4/+*, $P<0.05$; Student's *t*-test) at 120 h AEL following a 24-h induction in *dMyc* gene expression. (**C**) Immunoblotting of whole larval protein extracts with antibodies specific to Brf and β-tubulin revealed an increase in Brf protein levels following dMyc overexpression (using the flp-out technique) compared with controls at 120 h AEL following a 24-h induction in *dMyc* gene expression. (**D**) Co-immunoprecipitation studies revealed a specific association between Brf and dMyc proteins in wild-type (*yw*) *Drosophila* larval extracts 96 h AEL. (**E**) Fat body expression of dMyc (*cg>UAS-dMyc*) increases pupal volume compared with controls (*cg>+*). This effect is abolished when Brf levels are decreased in the larval fat body (*cg>UAS-brf RNAi, UAS-dMyc*) and pupae are similar in size to controls. *cg>brf RNAi* larvae fail to progress to the pupal stage. (**F**) Rapamycin treatment of *dMyc* mutants failed to decrease Pol III-dependent transcription further at 48 h AEL in whole larvae. (**G**, **H**) Overexpression of *dMyc* (using the flp-out technique) failed to reverse the decrease in tRNA synthesis in starved animals (**G**) and rapamycin-treated animals (**H**), as was measured by qRT–PCR of RNA extracted from whole larvae 72 h AEL. For all qRT–PCR experiments error bars indicate s.e.m.

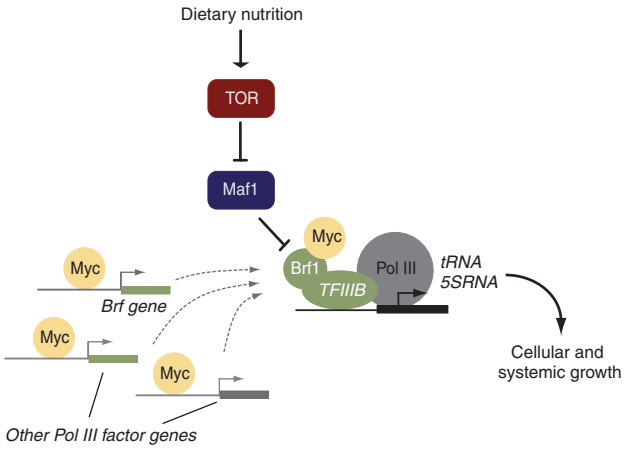

Figure 8 A model for nutrient/TOR regulation of Pol III in *Drosophila*. Our data suggest the predominant mechanism by which nutrition/TOR controls Pol III is via Maf1 repression, since Maf1 inhibition completely reverses the decrease in tRNA synthesis caused by TOR inhibition. Myc is sufficient and necessary for Pol III transcription, through controlling levels of Pol III factors (such as Brf) and through interaction with Brf. TOR can control Myc protein levels (Parisi *et al*, 2011; Teleman *et al*, 2008—dashed arrow in model figure). But these effects probably do not play a major role in how TOR activates Pol III since our data show that—unlike Maf1 inhibition—maintaining Myc levels and activity cannot reverse the decrease in tRNA synthesis caused by TOR inhibition.

TOR activity can be stimulated by extracellular cues such as growth factors, nutrients and oxygen (Wang and Proud, 2009) to control cell, tissue and organismal growth.

Despite the knowledge of the signalling inputs to TOR, we know little about the mechanisms that allow TOR to modulate cell metabolism and drive growth. Most studies on metabolic functions modulated by TOR have been confined to yeast and mammalian cell culture. These studies have been important in defining roles for TOR in protein synthesis, nutrient uptake and metabolism and autophagy (De Virgilio and Loewith, 2006; Wullschleger *et al*, 2006). But they leave open the question of what mechanisms operate *in vivo* to control tissue and organ growth during animal development. Genetic studies in *Drosophila* have been pivotal in this regard (Grewal, 2008; Hietakangas and Cohen, 2009; Teleman, 2009). Here, we show that the ability of the TOR pathway to control transcription through Pol III governs cell, tissue and ultimately organismal growth in *Drosophila*. Given that Pol III drives transcription of several non-coding RNAs required for mRNA translation, we suggest that the stimulation of Pol III by TOR enhances the protein synthetic capacity of cells. We previously showed that *Drosophila* TOR also controls synthesis of rRNA synthesis, via the RNA polymerase I factor, TIF-IA (Grewal *et al*, 2007). Moreover, recent studies in *Drosophila* larvae demonstrated that the insulin/TOR pathway regulates the expression of ribosome biogenesis genes via the transcription factors FOXO and Myc (Teleman *et al*, 2008; Li *et al*, 2010). Thus, in *Drosophila*, tissue and organismal growth relies on the ability of TOR to regulate all three nuclear RNA polymerases to ultimately promote protein synthesis. Given that regulation of all three polymerases is a conserved function for TOR, we suggest that these mechanisms may also underlie tissue and organ growth in mammalian development.

The Pol III transcription factor Brf is an essential component of the TFIIIB complex responsible for recruiting Pol III to gene promoters (Geiduschek and Kassavetis, 2001). Our work indicates that Brf activity is required for *Drosophila* development. Patterning and cell fate specification appear normal in *brf* embryos. However, once these mutants hatch as larvae they fail to grow. Our data suggest that this growth arrest phenotype reflects a role for Brf activity downstream of TOR. We found that Brf is cell-autonomously required for growth in both endoreplicating cells, which make up the bulk of larval mass, and the mitotically dividing cells of the imaginal discs. In particular, we find that *brf* mutant wing disc cell clones are outcompeted by wild-type neighbours. This cell competition phenotype is seen in mutants for other genes required for protein synthesis, such as the ribosomal proteins and Myc (Johnston, 2009). An important finding was that the overgrowth caused by loss of TSC1 (and hence increased TOR activity) was blocked in *brf* mutant cells. In mammalian cells, Brf activity is induced by cues that promote cell growth (e.g., during hypertrophic growth of cardiac cells) whereas cell differentiation leads to inhibition of Brf (Goodfellow and White, 2007; Athineos *et al*, 2010). In fact, overexpression of Brf alone can promote proliferation and transformation in immortalized fibroblasts (Marshall *et al*, 2008), while loss of Brf inhibits these processes (Johnson *et al*, 2007; Marshall *et al*, 2008). Mutations in tumour suppressors such as TSC are common in cancer and lead to elevated TOR activity and promotion of tumour growth. Based on our data, we suggest that Brf is required *in vivo* for both normal tissue growth and TOR-induced tumour growth.

Our data indicate the predominant mechanism by which nutrition/TOR controls Pol III is via Maf1 repression, since Maf1 inhibition completely reverses the decrease in tRNA synthesis caused by reducing TOR activity. These findings extend those observed in both yeast and mammalian cell culture, and suggest an important role for dMaf1 *in vivo* in developing tissues. The exact mechanism by which Maf1 functions is not clear, but it may involve inhibition of Brf and Pol III recruitment to genes, possibly by direct binding or association with Brf/Pol III (Desai *et al*, 2005; Vannini *et al*, 2010). Indeed, we could see an enhanced association between dMaf1 and Brf1 upon TOR inhibition. We also explored the role of dMyc as a potential link between nutrient-TOR signalling and Pol III. We found that dMyc was both necessary and sufficient for the control of Pol III activity during development. As previously reported in both mammalian and *Drosophila* culture, we were able to identify an interaction between dMyc and Brf (Gomez-Roman *et al*, 2003; Steiger *et al*, 2008). In addition, we identified a role for dMyc in controlling the levels of components of the Pol III machinery, including both Trf and Brf which form part of the TFIIIB complex. Thus, dMyc likely has both direct and indirect effects on Pol III activity in *Drosophila*. These effects are necessary for both dMyc-induced cell growth (Steiger *et al*, 2008) and, as we show here, for the non-autonomous increases in body size caused by dMyc in fat cells. Previous studies have shown that, in *Drosophila*, TOR controls Myc protein levels (Teleman *et al*, 2008; Parisi *et al*, 2011). But these effects on Myc probably do not play major role in how TOR activates Pol III since our data show that, unlike inhibition of Maf1, maintaining Myc levels and activity cannot reverse the decrease in tRNA synthesis caused by TOR

inhibition. Moreover, if Myc protein levels were limiting for TOR-dependent control of Pol III, then we would not expect that knockdown of Maf1 could completely reverse the effects of rapamycin/starvation. Given that Maf1 inhibition did not influence levels of Pol III factors, pre-rRNA or RP gene mRNA—transcripts that are upregulated by dMyc—it is unlikely that Maf1 influences Myc function. We did find that rapamycin feeding could not exacerbate the reduction of tRNA levels seen in dMyc null mutants. This result in principle may suggest that TOR signalling does not exert any dMyc-independent effects on Pol III function. But, we suggest this finding probably occurs because in the absence of Myc, Pol III activity may be approaching basal levels and cannot be significantly decreased much further. Taken together, although our data may not completely rule out some contribution of Myc to TOR-dependent control of Pol III, they do indicate that it is not the major contributor.

It is clear that both TOR and Myc are essential regulators of Pol III. But, it is likely that while TOR can control Myc levels, both TOR and Myc can also function in parallel and independently of each other. Teleman *et al* (2008) previously showed that overactivation of TOR signalling could not promote growth when Myc was inhibited, but at the same time Myc overexpression could not promote growth when TOR was inhibited. These findings and our data suggest that TOR and Myc cannot necessarily be placed in a simple, linear pathway. Recent studies in *Drosophila* have emphasized how other conserved growth-regulatory pathways, particularly those that control growth of the imaginal tissues (such as Wingless, EGF/Ras, the Hippo-Yorkie pathway and Bantam RNAi) function via control of dMyc (Johnston *et al*, 1999; Prober and Edgar, 2002; Herranz *et al*, 2011; Neto-Silva *et al*, 2011; Ziosi *et al*, 2011). Thus, dMyc may play a role in coupling these pathways to the control of Pol III activity to stimulate cell growth and proliferation.

It is interesting to speculate as to which Pol III targets are important for growth control. Pol III regulates the expression of several short non-coding RNAs, such as the tRNAs, 5S rRNA and 7SL RNA. Regulation of 5S rRNA production by Brf could influence ribosome synthesis and hence growth. However, we found that loss of Brf did not inhibit Pol I activity or alter levels of rRNA, suggesting that Brf probably does not directly influence ribosome numbers. One attractive possibility is that levels of the tRNAs may be limiting for translation and growth. In support of this notion, a recent paper showed that overexpression of Brf increased tRNA levels and promoted proliferation and transformation of cultured mammalian fibroblasts (Marshall *et al*, 2008). These effects of Brf were phenocopied by just increasing levels of $tRNA_i^{Met}$, and were associated with augmented mRNA translation and increased protein levels of growth promoters such as c-Myc and cyclin D1. We did not see a consistent increase in tRNAs when we simply overexpressed Brf in larvae, perhaps because levels of other components of the TFIIIB complex are limiting in flies. Nevertheless, by controlling Brf activity and tRNA synthesis, TOR could promote translation of growth regulators and drive larval growth. In fact, a recent paper indicated that TOR signalling in *Drosophila* regulates dMyc protein levels, but not *dMyc* mRNA levels, consistent with a possible role for translational control (Teleman *et al*, 2008).

One interesting result of our work was the identification of a non-cell autonomous role for Brf in organismal growth. Specifically, we found that Brf activity in the fat cells of *Drosophila* larvae could influence larval growth and final size. Elegant work by Leopold and colleagues has outlined a role for TOR in the fat body as a relay to control peripheral insulin signalling. In feeding larvae, amino-acid input into fat cells activates TOR, leading to transmission of a secreted signal from fat to brain to increase dILP expression and release from brain IPCs (Colombani *et al*, 2003; Geminard *et al*, 2009). Our data suggest that stimulation of Pol III activity may be an important downstream effector of this adipose function of TOR. Thus, adipose-specific silencing of Brf led to reduced peripheral insulin signalling, slower larval growth rate and reduced final body size. We found that, as in starved larvae, loss of *brf* led to reduced expression of *dilp* mRNA (seen in both *brf* mutants and *cg>brf RNAi* larvae) and reduced dILP release from the brain. Moreover, given that levels of phospho-Akt are lower, and levels of *dInR* (a FOXO target) are higher in tissues from both *brf* mutant and r4>brf *RNAi* larvae it is clear that systemic insulin signalling is reduced when Brf is inhibited in the fat body. We also found that another fat phenotype associated with starvation and loss of TOR, accumulation of lipid droplets, was phenocopied by loss of Brf. However, the autophagy phenotype of starved larval fat bodies was not phenocopied by loss of Brf. Therefore, Brf and Pol III function in the *Drosophila* fat body may mediate some, but not all of TOR's effects on growth and metabolism. The exact nature of the fat-to-brain secreted factor that controls insulin release in flies is not yet known, but perhaps translation of this signal, if it is a peptide or secreted protein, is influenced by changes in tRNA synthesis and translation rates. Indeed, Leopold *et al* showed that dMyc activity in the fat body was also important for controlling systemic insulin signalling, growth and body size (Delanoue *et al*, 2010). This effect of dMyc correlated with elevated expression of ribosome biogenesis genes and increased nucleolar size, an index of ribosome synthesis. We find that dMyc overexpression can also stimulate Pol III and tRNA levels, and that the increase in body size caused by fat body overexpression of dMyc is reversed by knockdown of Brf. These data suggest that regulation of mRNA translational capacity is a key step downstream of TOR and dMyc in fat cells to control signalling to IPCs.

Together, these data suggest that mRNA translational control may underlie a role for the fat body as an endocrine organ. A similar theme is emerging in mouse models. Mammalian adipose tissue is known to secrete adipokines and leptin to influence organismal metabolism and growth (Waki and Tontonoz, 2007). The secretion of many of these factors is influenced by diet, suggesting a regulatory role for TOR signalling. Genetic inhibition of either TOR and S6K in mice leads to alterations in metabolic activity in adipose tissue (Um *et al*, 2004, 2006; Polak *et al*, 2008; Cybulski *et al*, 2009). Moreover, loss of the translational repressors, 4E-BP1 and 4E-BP2, both of which are downstream TOR effectors, alters lipid and glucose metabolism in mice (Le Bacquer *et al*, 2007). To date, there are no mouse models of Pol III. However, it is interesting to speculate that changes in Pol III and tRNA synthesis are involved in mediating effects of TOR in adipose tissue in mice. Regulation of Pol III by TOR may also be important in the metabolic control of other processes. For example, TOR is a conserved regulator of organismal stress responses and lifespan (Kapahi *et al*,

2010). These stress responses rely on TOR's ability to control translation. We suggest that regulation of Pol III and tRNA synthesis may also be a mode of control. Further organismal studies, using genetic modulation of Pol III function, should provide additional insights into these points.

Materials and methods

Fly stocks

UAS-brf RNAi (NIG, Japan), $dtor^{\Delta P}$ (Zhang *et al*, 2000); $dS6K^{L1}$ (Teleman *et al*, 2005); UAS-$dS6K^{TE1}$, *UAS-dMaf1 RNAi*, *UAS-tsc1 RNAi/Tm6B*, *UAS-tsc1/2*, *UAS-dMyc*, *1-14-2* (Pierce *et al*, 2004); dm^4 (Pierce *et al*, 2004); $brf^{EY02964}$; *FRT82B,* $brf^{EY02964}$; *FRT82B,* $tsc1^{Q87X}$ (Tapon *et al*, 2001); *Df(3R)BSC565, da-GAL4, ptc-GAL4, ey-GAL4, r4-GAL4* (Lee and Park, 2004) and *cg-GAL4* were used (see FlyBase for further information: http://flybase.org).

All flies were reared and maintained at 25°C on standard *Drosophila* media (150 g agar, 1500 g cornmeal, 315 g yeast, 675 g sucrose, 1875 g D-glucose, 240 ml propionic acid per 34.5 l water).

Egg collection

Adult flies were allowed to lay eggs on grape juice agar plates supplemented with yeast paste for 4 h at 25°C. Twenty-four hours after egg laying (AEL), the plates were precleared of larvae and then larvae that hatched within the next 4 h were placed in food vials in groups of 50 and allowed to develop.

Starvation

Larvae were collected for starvation 72 h AEL and starved in sterile 20% sucrose in PBS, for 24 h unless stated otherwise in the figure legends. Following starvation, whole larvae were collected.

Rapamycin treatment of Drosophila S2 cells

Drosophila S2 cells (a kind gift from Edan Foley) were cultured at 25°C in Schneider's medium (Gibco; 11720-034) supplemented with 10% fetal bovine serum (Gibco; 10082-139), 100 U/ml penicillin and 100 U/ml streptomycin (Gibco; 15140). At 90% confluency, cells were treated with either 20 nM rapamycin (Calbiochem; 80054-246) or DMSO (Sigma; D2650) for 1 h, following which cells were washed twice with ice-cold PBS. Cells were then scraped into either TriZOL or protein lysis buffer (both procedures are detailed below) to prepare RNA and protein extracts, respectively.

Collection of material for RNA and protein extractions

Whole larvae or peripheral tissues were collected at the time points AEL as indicated in the figure legends. In the case of *r4>brf RNAi* experiments, peripheral tissues were prepared by stripping whole larvae of fat body. *dMyc* overexpression was performed using the heat-shock *flp-out* method (Elliott and Brand, 2008). For dMyc starvation and rapamycin treatment experiments, transgene expression was induced by incubating larvae at 37°C for 1, 48 h AEL. Controls lacking the *UAS* transgene were similarly heat shocked and treated as below. At 72 h AEL, fed control larvae were harvested while starved larvae were placed in 20% sucrose/PBS for a further 24 h after which they were also taken for RNA extractions. Rapamycin treatment took place in 35 mm petri dishes and involved placing larvae on a mixture of 3 g of prepared instant *Drosophila* media formula 4–24 (California Biologic Supply Company), 1 g of liquid inactivated yeast food and either 0.1% DMSO (Sigma; D2650) or 20 μM rapamycin (Calbiochem; 80054-246). Larvae were transferred to this food at 72 h AEL for a period of 24 h before being taken for RNA extractions. This rapamycin treatment method was used for the *dMyc* mutant and *dMaf1 RNAi* experiments at the times indicated above however in the case of the *dMyc* mutant experiments larvae were transferred to the DMSO/rapamycin containing food at 24 h AEL for a period of 24 h prior to RNA extractions. In each qRT–PCR experiment, a minimum of four groups of 8–10 larvae were collected. Each collection was independently performed a minimum of three times.

Quantitative RT–PCR

Total RNA was extracted using TRIzol according to manufacturer's instructions (Invitrogen; 15596-018). RNA samples (1 μg per reaction) were DNase treated according to manufacturer's instructions (Ambion; 2238G) and reverse transcribed using Superscript II (Invitrogen; 100004925). The generated cDNA was used as a template to perform qRT–PCRs (BioRad Laboratories; MyIQ PCR machine using SyBr Green PCR mix) using specific primer pairs (sequences available upon request). PCR data were normalized to the average fold change of either β-*tubulin1* or *tak1* mRNA levels, both of which were unchanged in response to a variety of environmental and genetic manipulations (Li *et al*, 2010). Each experiment was independently repeated a minimum of three times. All data were analysed by Student's *t*-tests.

Preparation of protein extracts, immunoblotting and antibodies

Whole larval protein extracts were prepared by washing material twice in ice-cold PBS before being homogenized in the appropriate volume of lysis buffer (20 mM Hepes (pH 7.8), 450 mM NaCl, 25% glycerol, 50 mM NaF, 0.2 mM EDTA, 0.5% Triton X-100, 1 mM PMSF, 1 mM DTT, 1 × Protease Inhibitor Cocktail (Roche, 04693124001)) using a motorized pestle. Following incubation on ice for 10 min, the lysates were cleared by centrifugation for 10 min at 10 000 r.p.m. at 4°C. *Drosophila* S2 cell extracts cells were prepared as previously described (Goodfellow and White, 2007). Protein (15 μg) was resolved by SDS–PAGE and immunoblotting performed as previously described (Marshall *et al*, 2008). Antibodies used were against a C-terminal fragment of *Drosophila* Brf (Takada *et al*, 2000), TBP (Santa Cruz Biotechnology Inc; 58C9), β-tubulin (E7, *Drosophila* Studies Hybridoma Bank), dMyc (Prober and Edgar, 2002) phospho-*Drosophila* Akt Ser505 (Cell Signaling Technology; 4054) and Akt (Cell Signaling Technology; 9272). Peptide antiserum against dMaf1 was raised by immunizing rabbits with synthetic peptide NNSQSGDEGITLC, corresponding to residues 74–87.

Immunoprecipitation

Drosophila S2 whole cell or larval extract (500 μg) was incubated at 4°C for 3 h on a rotating wheel with 25 μl protein A-sepharose beads (Sigma; P9424) that had been preincubated with antiserum against Brf, dMaf1, or a rabbit IgG control (Santa Cruz Biotechnology; sc-2027). Bound material was resolved by SDS–PAGE and specific proteins detected by immunoblotting as previously described (Marshall *et al*, 2008). Antibodies used for immunoprecipitation and immunoblot are described above.

Mitotic recombination, clone and cell size analysis

Mitotic recombination was performed using the *flp/FRT* method. For the fat body cell analysis, we performed a 6-h egg collection followed by a 1-h heat shock at 37°C. Larvae were transferred to food 24 h after heat shock. DAPI and phalloidin staining of fat bodies was performed on inverted and 4% paraformaldehyde fixed 120 h AEL larvae, following which fat bodies were dissected and mounted in Vectashield (Vector Laboratories Inc; H-1000). For analysis of twin-spot clones in the wing imaginal discs in Figure 3A–C, larvae were heat shocked for 20 min at 37°C, 60 h AEL. Wing discs were dissected at 120 h AEL and mounted in Vectashield for visualization. For Figure 1F clones were induced and wing discs were analysed at 24, 48 and 72 h after clone induction for counting. Viability of mutant clones in the wing imaginal discs was assessed by counting the percentage of wild-type clones that were still paired with a *brf* twin spot. Clone and cell sizes were calculated using Adobe Photoshop using the histogram tool. To induce wing disc clones for fluorescence-activated cell sorting (FACS) analysis, larvae were heat shocked at 37°C for 1, 72 h AEL and discs dissected and trypsinized 120 h AEL.

Flow cytometry

FACS analysis was performed on dissociated wing imaginal discs as previously described (Johnston *et al*, 1999).

Microscopy

All images were obtained on a Zeiss Observer Z1 microscope using Axiovision software. Microscopy and image capture were performed at room temperature and captured images were processed using Photoshop 7.0 (Adobe).

Nile red staining

Nile red staining of lipid droplets was performed as described previously (Grönke *et al*, 2005).

Lysotracker staining

Lysotracker staining was performed on dissected fat bodies from 72 h AEL larvae. Larval fat bodies were dissected in PBS then incubated in 1 μM Hoescht (Invitrogen; H3570), 100 μM lysotracker green (Molecular Probes; L7526) in 80% glycerol, on a coverslip for 15 min prior to image capture.

Pupation rates

Larvae were collected 24 h AEL and placed in food vials in groups of 50 per vial. The number of pupae formed on the side of the food vial was counted every 24 h and presented as a percentage of the total of number of pupae formed for each genotype.

Adult weight measurement

Following eclosion, adult flies were transferred to food vials in groups of 50 and aged for 3 days The average weight of adult males was calculated by weighing flies in groups of 10 with a precision balance (Sartorius). Data are presented as average weight calculated from at least five independent groups.

dILP2 immunostaining

dILP2 antibody staining of larval brains at 96 h AEL was as previously described (Geminard *et al*, 2009).

Pupal volume

Pupal volume was calculated as previously described (Delanoue *et al*, 2010).

Statistics

For all experiments, error bars represent s.e.m., and *P*-values are the results of a Student's *t*-test provided by Microsoft Excel.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: LM drafted and SG revised the manuscript. LM, ER and SG conceived and designed the experiments. LM, ER and SG performed the experiments, analysed the data and prepared the figures. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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