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

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Review timeline:

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 19 January 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. Firstly, apologies that the review process has taken a little longer than normal: this was largely due to the Christmas break, and referees consequently requiring longer to complete their reports. However, your manuscript has now been seen by three referees, whose comments are enclosed below. I'm sorry to say that the outcome is not a positive one. While all three referees recognise the interest in the topic under investigation, and while they differ in their overall level of support, all raise significant concerns with the study that would preclude publication EMBOJ at this point. Referees 1 and 2 both highlight the same main concern (most explicitly stated by referee 1), namely that your data do not elucidate the mechanism by which the TOR pathway regulates Pol III-dependent transcription. This is particularly important given that the basic principle - that Pol III transcription is regulated by TOR - has been demonstrated in other systems, and that mechanisms underlying this regulation have been established. Referee 3 also raises a number of critical concerns with the study, although we would not necessarily agree with this referee that your 2007 study on Pol I regulation by TOR compromises the novelty of the present work.

We do realise that you may well be able to tackle many of the more technical concerns without too much difficulty. However, addressing the question as to how Brf and/or Pol III function is regulated by TOR is clearly a much larger one. Our assessment - and that of the referees - is that significant novel insight into this question would be important for potential publication here. Since it is not clear that you will be able to do this, and particularly not within the time-frame of a normal revision, I am afraid we see little option other than to reject your manuscript at this stage. Given the high number of submissions we receive, we can only consider those which receive an enthusiastic report from at least a majority of the referees upon initial review. I am sorry we can not be more positive
on this occasion, but we hope that you find the referees' comments helpful when revising the manuscript for future submission elsewhere.

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

TOR kinase is a conserved regulator of cell and tissue growth. It is known to promote tissue growth cell autonomously by enhancing protein biosynthesis, by enhancing cap-dependent translation (e.g. through 4EBP and S6K) as well as by promoting ribosome biogenesis (e.g. through TIF-IA and Myc). Moreover, in Drosophila, TOR activity in the fat body has been shown to promote systemic growth through an unknown hormonal mechanism influencing secretion of insulin-like peptides.

The manuscript by Marshall and co-workers addresses the downstream effects of TOR signalling in the context of protein biosynthesis and growth control. The topic of the study is highly relevant and it is a natural continuation of previous work performed by Dr. Grewal. Technically the manuscript is of high quality, the experiments are well controlled and documented. The manuscript is well written and data is appropriately discussed in the context of earlier literature.

The authors show convincingly that nutrition-regulated TOR activity promotes the expression of several Pol III-dependent transcripts. The connection between TOR and Pol III has been earlier established in other systems, but this work confirms this connection in vivo. The authors also show that genetic ablation of Brf, a transcription factor limiting for Pol III transcription in yeast and mammals, leads to cell growth inhibition and downregulation of Pol III-dependent transcripts. brf is shown to be epistatic to tsc1, suggesting that Brf acts downstream of TOR. Finally, the authors show that RNAi-mediated knockdown of Brf in the fat body causes a starvation-like response with increase in lipid droplet size. Knockdown of fat body Brf causes a systemic growth inhibition, presumably through secretion of insulin-like peptides (as described earlier by the Leopold lab for TOR and Myc).

The main weaknesses in this study are somewhat limited novelty and lack of mechanistic insight. Many of the main findings, such as TOR-mediated regulation of Pol III transcripts and involvement of Pol III-mediated transcription in cell growth have already been shown in other model systems. The finding that Pol III-dependent transcription in fat body is involved in systemic growth control is novel and it extends the earlier findings of Leopold's group. The potential regulatory mechanism between TOR and Brf remains unexplored. Expanding the study substantially into this direction would significantly improve the manuscript.

Major comment:

1. The authors show evidence that both TOR and Brf regulate Pol III transcripts and that brf is epistatic to tsc1. This data is consistent with at least two alternative models: 1. TOR regulates the level or activity of Brf or 2. TOR regulates the level or activity of a third factor, which influences Pol III transcription in a Brf-dependent manner. There are two obvious candidates for the third factor, Maf1 and Myc, involvement of which ought to be explored. Maf1 is a repressor of Pol III transcription (Pluta et al 2001) and TOR has been shown to phosphorylate Maf1 and regulate its nucleolar localization (Wei et al. 2009, Shor et al., 2010). Drosophila Myc protein levels have shown to be regulated by TOR (Teleman et al. 2008) and in mammalian cells Myc promotes Pol III-dependent transcription and interacts with Brf (Gomez-Roman et al., 2003). In fact, the authors mention in the Discussion that they have unpublished evidence showing that Myc promotes Pol III-mediated transcription in Drosophila as well. Thus, I recommend the authors to significantly extend this study to test these alternative models. It should be relatively straightforward to analyze whether the expression level, phosphorylation or chromatin binding of Brf is regulated by TOR activity and/or whether Myc or Maf1 (or both) might constitute the regulatory link between TOR and Pol III.
(possibly in a Brf-dependent manner).

Minor comments:

2. In Figures 4 and 5 the authors use RNAi to knock down Brf. The key findings should be verified with an independent non-overlapping RNAi.

3. It is mentioned that effects similar to those of r4-GAL4 driver were observed using cg-GAL4, but the data is not shown. The data should be shown as supplemental figure.

References:


Referee #2

The authors explore the contribution of Pol III dependent transcription as an effector of insulin/TOR signaling in controlling growth of the model organism Drosophila. They provide evidence that TOR activity impacts on Pol III transcription. Furthermore, they demonstrate that Brf, a critical component of Pol III, is required for cellular growth. Interestingly, reducing the activity of Brf specifically in the fat body impinges on organismal growth, suggesting that Pol III mediated transcription in the fat body contributes to the non-autonomous regulation of insulin signaling. These findings are exciting and clearly add to our understanding of insulin/TOR signaling and growth control. However, there are some issues in need of clarification.

My main question concerns the relationship to Myc. In their analysis of Brf, the authors find a number of parallels to reported Myc phenotypes. Those similarities should be discussed and further investigated:

- Myc (diminutive) l.o.f. clones, like Brf l.o.f. clones, are out-competed (Johnston et al., 1999; de la Cova et al., 2004, Moreno and Basler, 2004).
- Myc protein levels are controlled by insulin/TOR signaling (Teleman et al., 2008), resembling the regulation of Pol III activity by insulin/TOR.
- Myc has been shown to impact on Pol III transcription (Gomez-Roman et al., 2006; Steiger et al., 2008).
- Myc activity in the fat body controls systemic insulin signaling (Delanoue et al., 2010).

Furthermore, Myc has been demonstrated to physically and genetically interact with Brf (Steiger et al., 2008). It is thus conceivable that insulin/TOR signaling impacts on Pol III via the control of Myc protein levels. In my view, it is essential to evaluate the role of Myc in linking insulin/TOR signaling with Pol III transcription.

Specific comments to the experiments:

- Figure 1:
The authors use starvation to downregulate TOR activity. Although it is well known that starvation reduces TOR activity, it is dangerous to claim that all effects of starvation are due to low TOR activity. Thus, a more direct testing of TOR is required. The only experiment that the authors performed in this regard (assessment of rRNA levels in TOR null mutant larvae; Figure 1E) is not satisfying. It would be useful to test an inducible system to manipulate TOR activity and to monitor the immediate consequences thereof (e.g., hs-Gal4 or tub-Gal4 tub-Gal80ts in combination with UAS-TOR-IR, UAS-Rheb-IR, or UAS-Tsc1/Tsc2, respectively). The same holds true for the opposite experiment (overactivation of insulin/TOR, Figure 1F). An inducible system should be
used to drive UAS-Rheb or UAS-Tsc1-IR (which more directly activate TOR than overexpression of Dp110 does).

- Figures 2, S2, 3:
  What is the contribution of apoptosis to the effects observed in clones of cells lacking Brf in wing imaginal discs? Does blocking apoptosis, e.g. by the expression of the caspase inhibitor p35, impact on clone size? This is especially relevant for the Tsc1 Brf double mutant analysis. The conclusion that Brf is required for TOR-driven growth is only justified if the small clone size is not simply a consequence of massive cell death.

- Figure 5:
  A more direct readout for in vivo insulin signaling activity should be used (e.g. tGPH to monitor PIP3 levels at the membrane, or phospho-Akt antibody staining to monitor the activation of Akt).

Minor comments:

- I do not understand the normalization that has been used in the qRT-PCR experiments. In Figures 1A, 1C, 2B, 5D, 5E, 5F and S1, the normalized values of the control conditions deviate from 1. How can this be explained?

- The scale bars are not defined in Figure 3.

- There is no blue signal in Figures S2D and S2E.

Referee #3

In this manuscript, Marshal et al explore a possible regulation of RNA polIII transcription by TOR signalling in Drosophila. They show that scarce food has a negative effect on the expression of RNA pol III transcripts, similar to the effect seen in TOR mutants. They study Brf, a conserved component of the TFIIIB, limiting for Pol III transcription in yeast and Drosophila. Using epistatic analysis, they propose that brf is required for TOR-induced cell growth. Finally, they show that in the fat body, brf loss of function has similar effects on lipid metabolism to those observed upon starvation. Concomitantly, they observe that brf silencing in the fat body suppresses peripheral insulin signalling and therefore systemic growth. In conclusion, they propose that in response to nutrition, TOR signalling controls cell growth and systemic growth by driving brf expression and thus Pol III transcription in the fat body.

The general topic is of broad interest. Nevertheless, the study is incomplete in its current state. A paper published in 2007 by the group already referred to the connection between TOR and rRNA synthesis. In comparison, the present work does not provide sufficient novelty to merit publication in EMBO J.

Specific comments:

Figure 1
The first part of the result section is not clear. What is the difference between figure 1A and 1B. Are 20% sucrose/PBS (no agar?) and 20% agar different starvation conditions? What is the duration of starvation in figure 1B? t-test significance is unclear since it is a pair wise statistical test that is calculated here for 3 conditions (fed, 24hr starved and 48hr starved in figure1A or Fed, Agar + 20% sucrose, Agar in Figure 1B). In the body text, it is unclear whether Pol III-dependant transcripts and Pol III transcripts refer to the same category of transcripts. Preparation of muscle tissue extract is not described in the material and method section.

Is overexpression of PI3K sufficient to activate TOR signaling in Drosophila? Schleich and Teleman (2009) have shown that phosphorylation of TSC1 and 2 by Akt is dispensable for growth in this system. Other tools, more specific of the TOR pathway itself, such as Rheb or S6K overexpression, or TSC1/2 silencing, need to be tested in the same conditions to draw solid conclusion on the regulation RNA Pol III-dependant transcripts by TOR.
Levels of the 5S rRNA and 7SL RNA should be shown in the brf mutant background. Are RNA pol I transcripts affected?

In figure 2E, were experiments done using imaginal tissues? Was it measured on surviving clones? If so, why not performed for the FACS analysis rather than using heterozygous conditions?

Cell doubling time should be calculated to conclude that cells are indeed dividing at slower pace.

Figure 3

This aspect is central to the manuscript but needs to be strengthened. Removing brf has a drastic effect on cell size and viability. Is it possible to rescue such a strong phenotype by simply removing tsc1, given that the lack of Tsc1 has only a moderate effect on cell size (20% increase)? Is this the strongest condition to activate the TOR pathway? Rheb or S6K overexpression should be evaluated in the same conditions.

Is this effect specific for the TOR pathway? Are other growth inducers (Myc, Ras, PI3K/AKT, CyclinD/cdk4) able to rescue brf cell size defects?

Does Tsc1/2 loss of function, or Rheb or S6K overexpression, rescue the decrease of RNA Pol III transcripts both in mitotic and endoreplicative tissues?

Figure 5

Does brf overexpression in the fat body influence both systemic growth and peripheral insulin signaling? What is the effect of this overexpression in starved conditions?

What is the effect of R4>brf RNAi on brain dilp expression/levels?

Minor comments

Page 5: Abbreviations such as brf should be defined.
Page 17: The egg collection part needs to be clarified.

Figure legend S1: rapamycin is an inhibitor of the TOR pathway, not insulin pathway.

Figure 3 legend : panel 3J does not exist.

Figure legend 5: as far as I understand, t-test was calculated using n=3 and not n=32.

Resubmission 13 August 2011

Response to Reviewers:

Responses to Reviewer 1.

Major comment:

The authors show evidence that both TOR and Brf regulate Pol III transcripts and that brf is epistatic to tsc1. This data is consistent with at least two alternative models: 1. TOR regulates the level or activity of Brf or 2. TOR regulates the level or activity of a third factor, which influences Pol III transcription in a Brf-dependent manner. There are two obvious candidates for the third factor, Maf1 and Myc, involvement of which ought to be explored. Maf1 is a repressor of Pol III transcription (Pluta et al 2001) and TOR has been shown to phosphorylate Maf1 and regulate its nucleolar localization (Wei et al. 2009, Shor et al., 2010). Drosophila Myc protein levels have shown to be regulated by TOR (Telemann et al. 2008) and in mammalian cells Myc promotes Pol III-dependent transcription and interacts with Brf (Gomez-Roman et al., 2003). In fact, the authors mention in the Discussion that they have unpublished evidence showing that Myc promotes Pol III-mediated transcription in Drosophila as well. Thus, I recommend the authors to significantly extend this study to test these alternative models. It should be relatively straightforward to analyze whether the expression level, phosphorylation or chromatin binding of Brf is regulated by TOR activity and/or whether Myc or Maf1 (or both) might constitute the regulatory link between TOR and Pol III (possibly in a Brf-dependent manner).

We thank the reviewer for his/her comments and constructive thoughts. Reviewer 1 makes the important point that there are at least two possible mediators of nutrient/TOR effects on Pol III – inhibition of the repressor Maf1 and activation of Myc. No studies have, however, directly compared or investigated contribution of either of these two factors in vivo in developing tissues.
We have now followed the Reviewer's suggestion and examined the role of both Maf1 and Myc in the control of Pol III by nutrient/TOR signaling. These new data have greatly helped us improve our manuscript. We discuss our main findings:

First, we find that Maf1 is the main link between nutrient/TOR signaling and Pol III transcription. We show that the decrease in Pol III activity in developing larvae by both starvation and rapamycin feeding is reversed by RNAi-mediated knockdown of dMaf1. We further show that inhibition of TOR (either starvation in larvae or rapamycin treatment in cultured S2 cells) doesn’t affect Brf protein levels. Rather we show that association between Maf1 and Brf is increased under conditions of TOR inhibition. This result points to a role for Maf1 in sequestering Brf when nutrient/TOR signaling is inhibited. These new data are presented in Fig 6.

Second, we show that dMyc plays a minor role downstream of nutrient-TOR signaling in the control of Pol III. We show that Myc is necessary and sufficient to stimulate Pol III in larvae. Moreover, we present two mechanisms that explain these effects. First we show that Myc can associate with Brf in larvae. This result is consistent with previous cell culture work and points to a role for Myc at Pol III-regulated genes. Second we show that Myc controls the expression levels of components of the Pol III machinery, including Brf and Trf, two proteins of the TFIIIB complex. This is the first report that Myc can control the levels of the Pol III machinery. But in contrast to our findings with Maf1, we show that Myc effects on Pol III are largely independent of TOR. Thus when we maintain high Myc levels, we cannot reverse the inhibition of Pol III-dependent transcription caused by nutrient starvation, and we only see a small increase in tRNA levels [Compare this result with the similar experiment in which we knockdown Maf1 and see a complete reversal of the effects of starvation - Fig. 6]. Importantly, we show that Myc can still induce a known direct target (ppan) in starved larvae, confirming that Myc is still functional in starved larvae. These new data are presented in Fig 7.

These data provide the first in vivo examination of how nutrient/TOR signaling regulates Pol III. By using a genetic approach to directly examine and compare the effects of dMaf1 and dMyc, we provide important new insights into how the nutrient/TOR pathway functions in developing animals.

Minor comments:

2. In Figures 4 and 5 the authors use RNAi to knock down Brf. The key findings should be verified with an independent non-overlapping RNAi.

We didn’t have another non-overlapping Brf RNAi construct. Instead we rescued the growth inhibitory effects of our RNAi construct with overexpression of UAS-Brf. This result suggests our UAS-RNAi line specifically targets Brf. These data are presented in Supplemental Figure 2.

3. It is mentioned that effects similar to those of r4-GAL4 driver were observed using cg-GAL4, but the data is not shown. The data should be shown as supplemental figure.

We’ve added this data as Supplemental Figure 4.

Responses to Reviewer 2

My main question concerns the relationship to Myc. In their analysis of Brf, the authors find a number of parallels to reported Myc phenotypes. Those similarities should be discussed and further investigated:

- Myc (diminutive) l.o.f. clones, like Brf l.o.f. clones, are out-competed (Johnston et al., 1999; de la Cova et al., 2004, Moreno and Basler, 2004).
- Myc protein levels are controlled by insulin/TOR signaling (Teleman et al., 2008), resembling the regulation of Pol III activity by insulin/TOR.
- Myc has been shown to impact on Pol III transcription (Gomez-Roman et al., 2006; Steiger et al., 2008).
- Myc activity in the fat body controls systemic insulin signaling (Delanoue et al., 2010). Furthermore, Myc has been demonstrated to physically and genetically interact with Brf (Steiger et al., 2008). It is thus conceivable that insulin/TOR signaling impacts on Pol III via the control of Myc
protein levels. In my view, it is essential to evaluate the role of Myc in linking insulin/TOR signaling with Pol III transcription.

We thank the reviewer for their constructive review, and we appreciate their comments and thoughts on improving the manuscript. We agree with the reviewer that the phenotypes of Brf are similar to Myc phenotypes, and we briefly comment on this in the Discussion section. Therefore, as suggested by the reviewer (and also by reviewer 1) we have evaluated the role of Myc in linking TOR signaling to Pol III. As discussed in our comments to Reviewer 1, we show that dMyc plays a minor role downstream of nutrient-TOR signaling in the control of Pol III. We show that Myc is necessary and sufficient to stimulate Pol III in larvae. Moreover, we present two mechanisms that explain these effects. First we show that Myc can associate with Brf in larvae. This result is consistent with previous cell culture work and points to a role for Myc at Pol III-regulated genes. Second we show that Myc controls the expression levels of components of the Pol III machinery, including Brf and Trf, two proteins of the TFIIIB complex. This is the first report that Myc can control the levels of the Pol III machinery. But in contrast to our findings with Maf1, we show that Myc effects on Pol III are largely independent of TOR. Thus when we maintain high Myc levels, we cannot reverse the inhibition of Pol III-dependent transcription caused by nutrient starvation, and we only see a small increase in tRNA levels [compare this result with the similar experiment in which we knockdown Maf1 and see a complete reversal of the effects of starvation - Fig. 6]. Importantly, we show that Myc can still induce a known direct target (ppan) to same extent in both fed and starved larvae, confirming that Myc is still functional in starved larvae. These new data are presented in Fig 7.

Thus we suggest that Myc and TOR-Maf1 signaling represent two separate inputs to the control of Pol III during tissue and organismal growth. We discuss these findings in detail in the Discussion section of the paper.

Specific comments to the experiments:

- Figure 1:
The authors use starvation to downregulate TOR activity. Although it is well known that starvation reduces TOR activity, it is dangerous to claim that all effects of starvation are due to low TOR activity. Thus, a more direct testing of TOR is required. The only experiment that the authors performed in this regard (assessment of tRNA levels in TOR null mutant larvae; Figure 1E) is not satisfying. It would be useful to test an inducible system to manipulate TOR activity and to monitor the immediate consequences thereof (e.g., hs-Gal4 or tub-Gal4 tub-Gal80ts in combination with UAS-TOR-IR, UAS-Rheb-IR, or UAS-Tsc1/Tsc2, respectively). The same holds true for the opposite experiment (overactivation of insulin/TOR, Figure 1F). An inducible system should be used to drive UAS-Rheb or UAS-Tsc1-IR (which more directly activate TOR than overexpression of Dp110 does).

We appreciate the Reviewer's comments about TOR signaling – similar issues were also raised by Reviewer 3. We have therefore added several new pieces of data:

- Firstly, we find that feeding larvae the TOR inhibitor rapamycin leads to reduced tRNA synthesis (Fig 6A-F). Together with similar cell culture data (Suppl Fig 5), these pharmacological data argue that TOR controls Pol III in flies.

- Secondly, we complement our starvation and TOR mutant data by showing that expression of UAS-Tsc1/Tsc2 can suppress tRNA levels in larvae (Fig 4C).

- Finally we also pinpoint role for S6K downstream of TOR. We use both gain-of-function (Gal4/UAS expression) and loss-of-function (S6K mutants) to show that S6k regulates Pol III in larvae (Fig 4D, F).

Together these pharmacological and genetic data strongly support a role for TOR-S6K in controlling Pol III in flies.

- Figures 2, S2, 3:
What is the contribution of apoptosis to the effects observed in clones of cells lacking Brf in wing imaginal discs? Does blocking apoptosis, e.g. by the expression of the caspase inhibitor p35, impact
Reviewers raises an important point – are the brf mutant cell clones small simply due to intrinsic cell death in the mutant cells, or is there a primary growth/proliferation defect in brf wing disc cells, which then leads to elimination of these cells by cell competition?

We present several pieces of data to support the latter, cell competition explanation:

- First, we did the experiment the reviewer suggested: we showed that overexpression of p35 could rescue the loss of viability of brf mutant cells clones. Thus, at 72hr post clone induction – when brf mutant cells are almost completely eliminated – we find that brf mutant cell clones overexpressing p35 are viable. But importantly, these brf+/p35 clones are still much smaller than wildtype clones. This result suggests that the primary phenotype in brf mutant cells is reduced growth/proliferation. These data are presented in Fig S3.

- Second, we showed that we could also rescue the death of brf mutant cells by using the Minute technique. Thus we rescued brf mutant clones by making the surrounding wt cells heterozygote for a dominant allele of a ribosomal protein, and hence reducing their growth rate. This is a classic approach to rescue mutant cells from cell competition and provides further evidence that the primary defect in the brf mutant cells is reduced growth leading to their elimination by surrounding faster growing cells.

- Finally, we see that in situations where cell competition is not observed (e.g in larval polyploid cells) loss of brf (either by RNAi or in genetic mutants) does not lead to any evidence of cell death (e.g pyknotic or fragmented nuclei – Fig 1D, 5D, S2E, F). This observation argues against a general cell-intrinsic death caused by reduced Brf function. Similarly, if we inhibit Brf in an entire compartment in the wing disc (e.g. in en-GAL4/UAS-Brf RNAi larvae), and hence do not set up a cell competition scenario, we don’t see any cell death as visualized by cleaved caspase 3 staining (data not shown).

- Figure 5:
A more direct readout for in vivo insulin signaling activity should be used (e.g. tGPH to monitor PIP3 levels at the membrane, or phospho-Akt antibody staining to monitor the activation of Akt).

The reviewer makes a very good suggestion. We have now carried out phospho-Akt westerns and we report reduced phospho-Akt in both brf mutants and in peripheral tissues of r4>brf RNAi larvae. We feel that these data together further strengthen our conclusion that fat body loss of Brf suppresses systemic insulin. These data have greatly improved the manuscript and we thank the reviewer for the suggestion.

Minor comments:

- I do not understand the normalization that has been used in the qRT-PCR experiments. In Figures 1A, 1C, 2B, 5D, 5F and S1, the normalized values of the control conditions deviate from 1. How can this be explained?
The data are not displayed as fold changes (with control at 1). Rather we express the data as relative changes calculated after normalizing for tubulin mRNA levels.

- The scale bars are not defined in Figure 3.
We apologize for this error. We have now given the scale bar information in the figure legend.

- There is no blue signal in Figures S2D and S2E.
We apologize for this error. The image is black and white.
comments below:
Specific comments:

Figure 1
The first part of the result section is not clear. What is the difference between figure 1A and 1B. Are 20% sucrose/PBS (no agar?) and 20% agar different starvation conditions?

In the original Fig 1A we starved larvae using a common procedure of floating the larvae on 20% sucrose in PBS. In the original Fig 1B we confirmed that the effects we see are due to nutrient deprivation and not simply high sucrose by now growing the larvae on agar (no nutrients) both with and without sucrose. The essential finding is the same – starvation suppresses Pol III. We’ve now simplified this presentation by just showing the fed/starved data from the original Fig 1A (now presented in Fig 4A).

What is the duration of starvation in figure 1B?

The duration is 24hrs.

t-test significance is unclear since it is a pair wise statistical test that is calculated here for 3 conditions (fed, 24hr starved and 48hr starved in figure1A or Fed, Agar + 20%sucrose, Agar in Figure 1B).

The reviewer is correct – we should use ANOVA analysis. We have, however, now removed this piece of data to simplify the presentation of the effects of starvation on Pol III activity (Fig 4).

In the body text, it is unclear whether Pol III-dependant transcripts and Pol III transcripts refer to the same category of transcripts.

We apologize for this confusion. For accuracy, we now refer to “Pol III-dependent transcripts” throughout the paper.

Preparation of muscle tissue extract is not described in the material and method section.

We have now added this information to the Methods section

Is overexpression of PI3K sufficient to activate TOR signaling in Drosophila? Schleich and Telean (2009) have shown that the phosphorylation of TSC1 and 2 by Akt is dispensable for growth in this system. Other tools, more specific of the TOR pathway itself, such as Rheb or S6K overexpression, or TSC1/2 silencing, need to be tested in the same conditions to draw solid conclusion on the regulation RNA Pol III-dependant transcripts by TOR.

We appreciate the reviewer’s comments about TOR signaling – similar issues were also raised by reviewer 2. We have therefore added several new pieces of data:

- Firstly, we find that feeding larvae the TOR inhibitor rapamycin leads to reduced tRNA synthesis (Fig 6A-F). Together with similar cell culture data (Suppl Fig 5), these pharmacological data argue that TOR controls Pol III in flies.
- Secondly, we complement our starvation and TOR mutant data by showing that expression of UAS-Tsc1/Tsc2 can suppress tRNA levels in larvae (Fig 4C).
- Finally we also pinpoint role for S6K downstream of TOR. We use both gain-of-function (Gal4/UAS expression) and loss-of-function (S6K mutants) to show that S6k regulates Pol III in larvae (Fig 4D, F).

Together these pharmacological and genetic data strongly support a role for TOR-S6K in controlling Pol III in flies.

Figure 2
Levels of the 5S rRNA and 7SL RNA should be shown in the bfr mutant background. Are RNA pol I transcripts affected?
We’ve now included this extra information in Figure S1. Levels of 7SL, another Pol III-dependent transcript, are reduced in \textit{brf} mutants. In contrast levels of 5S rRNA are unaffected, probably due to the well-described stability of 5S rRNA. We saw no change in pre-rRNA.

In figure 2E, were experiments done using imaginal tissues? Was it measured on surviving clones? If so, why not performed for the FACS analysis rather than using heterozygous conditions?

The expts in Fig 2E (now Fig 1E) were performed on wing imaginal discs – we have emphasized this is the text and legend. In this experiment we used \textit{flp/FRT}-mediated mitotic recombination to induce twospot clones (wt and \textit{brf} mutant clones). We then measured clone areas 48 h later. The data represent the average clone areas of wild-type and \textit{brf} mutant clones, and we find that \textit{brf} mutant clones are much smaller than wild-type. All the measurement are made on surviving clones. We’re a little confused by the reviewer’s question: “If so, why not performed for the FACS analysis rather than using heterozygous conditions”; Our experiments - measurement of clone area and cell size (by FACS) - involved comparing homozygote mutant cells (marked by absence of GFP) vs wild-type cells (marked by GFP).

Cell doubling time should be calculated to conclude that cells are indeed dividing at slower pace.

In our \textit{flp/FRT} experiments, we didn”t have the necessary lines to positively mark both wild-type and mutant cells. But our data indicate that the \textit{brf} mutant clones are smaller than wild-type, but the cell size is unchanged. Thus, by inference, there must be fewer cells in the \textit{brf} clones, which is consistent with reduced division rate. Furthermore, when we rescued the \textit{brf} mutant cells by overexpressing p35, the clones were still much smaller with clearly fewer numbers of cells than wt clones (Fig S3). Again, the straightforward inference is that the \textit{brf} mutant cells must have grown and divided at a slower rate than wt cells.

\textbf{Figure 3}

This aspect is central to the manuscript but needs to be strengthened. Removing \textit{brf} has a drastic effect on cell size and viability. Is it possible to rescue such a strong phenotype by simply removing \textit{tsc1}, given that the lack of \textit{Tsc1} has only a moderate effect on cell size (20% increase)? Is this the strongest condition to activate the TOR pathway? Rheb or S6K overexpression should be evaluated in the same conditions. Is this effect specific for the TOR pathway? Are other growth inducers (Myc, Ras, PI3K/AKT, CyclinD/cdk4) able to rescue \textit{brf} cell size defects? Does \textit{Tsc1}/2 loss of function, or Rheb or S6K overexpression, rescue the decrease of RNA Pol III transcripts both in mitotic and endoreplicative tissues?

The main goal of this experiment was to test the epistatic relationship between \textit{tsc1} and \textit{brf} – i.e. does Brf function downstream of TSC-TOR? We weren’t necessarily trying to „rescue“ the effects of \textit{brf1} by \textit{tsc1} mutants.

Loss of TSC is a strong activator of TOR – the papers describing \textit{tsc1} mutant phenotypes (Potter et al, Cell, 2001; Tapon et al,Cell, 2001; Gao and Pan, Genes Dev, 2001) report very strong overgrowth effects. These are certainly as strong as Rheb overexpression (Stocker et al, 2003, Nat Cell Biol; Saucedo et al, 2003, Nat Cell Biol) and much stronger than S6K overexpression (Kozma et al, 1999, Science). Importantly, \textit{tsc1} mutants can rescue strong growth inhibitory effects. Thus, loss of \textit{tsc1} rescues the pronounced growth inhibitory effects seen in both \textit{inr} and \textit{akt} mutant cells (Gao and Pan, 2001, Genes Dev). This analysis was used to indicate that \textit{tsc1} is epistatic to both \textit{inr} and \textit{akt}. In contrast, \textit{tsc1} mutants don’t rescue \textit{rheb} mutant cells, leading to the inference that \textit{rheb} is downstream of \textit{tsc1} (Stock er et al, 2003, Nat Cell Biol). Hence the main goal of our experiments in Fig 3 was to perform similar a type of epistatic analysis with \textit{brf} and \textit{tsc1}.

Another important reason for our approach concerns the genetics involved - both \textit{tsc1} and \textit{brf} (like \textit{akt}, \textit{inr} and \textit{rheb}) all are located on the right arm of the 3rd chromosome. Hence we can use genetic recombination to make single and double mutant cells. This is a very „clean“ and powerful genetic technique that highlights the strength of the Drosophila system to perform clonal analyses in developing tissue. This type of classic double mutant analysis is one of the most rigorous and powerful genetic approaches to examine epistatic relationships between genes.

\textbf{Figure 5}
Does brf overexpression in the fat body influence both systemic growth and peripheral insulin signaling? What is the effect of this overexpression in starved conditions?

We find that Brf overexpression has no effect on Pol III-dependent transcription in either fed or starved larvae. As such, we see no effects on cell or organismal growth (we have referred to this negative data as „data not shown“ in the manuscript).

What is the effect of R4>brf RNAi on brain dilp expression/levels?

As suggested by the reviewer, we have explored whether the decrease in systemic insulin following Brf knockdown might be due to decreases in mRNA expression of brain dILPS. Previous reports have shown that mRNA levels of dILP5, but not dILP2, are suppressed upon starvation (Geminard et al., 2009, Cell Metab). We also find that dILP5 mRNA levels are reduced in brf mutants (Fig 2H). By contrast, we see little difference in dILP mRNA expression levels in r4>brf RNAi larvae (Fig 2I). But this may be due to the fact that the effects of RNAi-mediated brf knockdown in the fat body may not influence fat-to-brain signaling as strongly as brf mutants and starved larvae. Indeed, the growth effects of brf knockdown in the fat body (slow growth, reduced body size) are weaker than the phenotype of brf mutants and nutrient-deprived larvae (complete growth arrest). Nevertheless, given that levels of phospho-Akt are lower (Fig 2D, E), and levels of dInR (a FOXO target) are higher in tissues from both brf mutant and r4>brf RNAi larvae (Fig 2F, G) it is clear that systemic insulin signaling is reduced in both cases. Hence, we suggest that the main effects of Brf knockdown on systemic insulin are probably due to changes in dILP release rather than just changes in dILP mRNA expression levels. Leopold and colleagues report a similar effect on dILP release in the context of nutrient deprivation and altered TOR signaling in the fat body (Geminard et al., 2009, Cell Metab).

Minor comments

Page 5: Abbreviations such as brf should be defined.
We have now defined Brf (TFIIIB-related factor) in the text.

Page 17: The egg collection part needs to be clarified.
We have now clarified how we collected eggs.

Figure legend S1: rapamycin is an inhibitor of the TOR pathway, not insulin pathway.
We have corrected this in the text.

Figure 3 legend: panel 3J does not exist.
We have corrected this in the text.

Figure legend 5: as far as I understand, t-test was calculated using n=3 and not n=32.
We have corrected this in the text.
will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1:

According to my suggestion the authors have expanded the study towards mechanistic understanding of the regulatory connection between TOR and PolIII-mediated transcription. While the study has much improved, I still have some concerns that should be addressed prior to publication.

1. The authors show that overexpression of Myc is not sufficient to significantly rescue the inhibitory effects of starvation on PolIII-mediated transcription. From this, the authors conclude that Myc plays only a minor role in mediating the effect of TOR to PolIII transcription. I think the evidence provided by the authors is not sufficient to justify such conclusion. To test whether Myc is essential in mediating the effects of TOR to PolIII, the authors should test the effect of rapamycin on PolIII targets in Myc null background. The current evidence showing that Myc is not sufficient allows alternative conclusions to be made, e.g. that TOR regulates several factors in PolIII transcription and restoring Myc is not enough compensate for the others.

2. As new data related to the downstream effectors have been added, I recommend the authors to revise the abstract to better reflect the revised content of the study.

Referee #2

In their revised manuscript, the authors (a) have included relevant new data concerning the roles of Maf1 and Myc in the regulation of Pol III-dependent transcription, (b) have adequately addressed most of the concerns raised by the reviewers, and (c) present their data in a much clearer way. This extended study provides significant new insights in the downstream effectors of TOR in growth regulation, which is certainly interesting to a broad readership. However, some issues need to be addressed before publication.

Specific comments

- It is questionable whether the use of Dp110 overexpression is ideal to substantiate the claim that increased TOR signaling results in an increase in Pol III-dependent transcription (Figure 4E). The authors do show that overexpression of an activated variant of S6K yield similar effects. Nevertheless, the use of UAS-Rheb (or UAS-Tsc1/2 RNAi) would be a more direct means to activate TORC1. It could be tested whether the effects of Dp110 overexpression are suppressed by reducing TOR function (genetically or by Rapamycin treatment) or in an S6K mutant background.
- The epistasis analysis shown in Figure 5 is compelling in the fat body but not in the wing imaginal disc. As previously stated, the conclusion that Brf is required for TOR-driven growth is only justified if the small clone size is not simply a consequence of apoptosis. Therefore, the experiment should be repeated in the presence of p35.

- The new data on the role of Maf1 substantially refine the picture of how TOR acts on Pol III activity. However, all the experiments are based on a single RNAi line that is neither specified nor characterized. The authors need to show the specificity of the RNAi effect.

- Is Maf1 phosphorylated upon high TOR activity?

- To clarify the contribution of Myc downstream of TOR in the regulation of Pol III-dependent transcription, the authors added a series of experiments shown in Figure 7. They find that Myc overexpression under starvation conditions does not restore normal levels of tRNA(Met) expression, and they conclude that Myc is therefore unlikely to represent a link between TOR and Pol III-dependent transcription. This conclusion is problematic for several reasons: (1) The authors only show effects on tRNA(Met). (2) The activity of Myc towards Pol III-dependent transcription could be decreased upon starvation (although Myc's function in Pol II-dependent transcriptional regulation appears to be unaffected). (3) Starvation is not equivalent to reduced TOR activity. To be more specific, the authors should reduce TOR activity by genetic means (or by Rapamycin treatment).

- Does TOR overexpression further increase the effects of Maf1 RNAi on Pol III-dependent transcription?

- Reducing brf function in the fat body by means of r4>brf RNAi impacts on systemic insulin signaling but it does not decrease dlip5 mRNA expression (Figure 2I; in contrast to the brf mutant situation shown in Figure 2H). Did the authors check other fat body drivers? Furthermore, the release of brain dILPs should be analyzed.

- Does Myc overexpression in the fat body still produce systemic growth effects when brf function is reduced?

- Figure 1E, Figure 2B: The unit mm2 is definitely wrong. In the legend to Figure 2C, it should be indicated that the weight of 10 adult males is displayed.

- Does the title convey the key message? Non-experts will not grasp that Brf, a subunit of TFIIIB, is essential for Pol III-dependent transcription. I would suggest to emphasize that TOR regulates Pol III activity.

General comments

- The genetic analysis of brf is not very clean. The brf locus is rather complicated as it contains two other genes in an intron (lute and CG43196). The EY02964 allele is probably not a null allele and is likely to also affect lute. Furthermore, the EY insertion is an EP element that drives expression of lute in the presence of Gal4. Did the authors test other transposon insertions in the locus? Is the severity of the phenotype enhanced when the transposon is placed over a deficiency? Of course, it is reassuring that brf RNAi yields similar results, and that the RNAi effects can be rescued by co-expression of brf. Getting the same effects with different RNAi lines (which are available) would further support the conclusions. The RNAi line used in this study should be defined.

- Throughout the manuscript, the authors use the term 'TOR activity'. I guess they refer to TORC1 activity exclusively. It might be interesting to check whether TORC2 also impacts on Pol III-dependent transcription.
**Editor Comments.**

As you will see, both reviewers find the manuscript substantially improved, and are now supportive of publication, pending some further revision. Their reports are explicit, but I would in particular point out the concerns raised by both referees as to the conclusiveness of the data on the contribution of Myc.

We thank the editor for the comments. We have now fully addressed the issues raised by both reviewers, particularly concerning the contribution of dMyc to TOR regulation of Pol III. We discuss our new data below.

**Referee #1:**

We thank the Reviewer for his/her support for our work and for helpful comments and suggestions on how to improve the manuscript.

According to my suggestion the authors have expanded the study towards mechanistic understanding of the regulatory connection between TOR and PolIII-mediated transcription. While the study has much improved, I still have some concerns that should be addressed prior to publication.

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Both Reviewer 1 and 2 raised some points concerning the link between TOR, Myc and Pol III. We have now performed all the additional experiments suggested by both reviewers.

In particular, we carried out the important experiment suggested by Reviewer 1 – we examined tRNA levels in rapamycin-fed dMyc null larvae. These data are presented in Fig. 7F and show that rapamycin feeding does not exacerbate the reduction in tRNA levels seen in dMyc null animals. This result in principle may suggest that TOR signaling does not exert any dMyc-independent effects on Pol III function. But unlike inhibition of dMaf1, maintaining high levels of dMyc was not sufficient to bypass the decrease in tRNA synthesis in either starved or rapamycin larvae (Fig 7G, H). Thus, dMyc may not be exclusively downstream of nutrient-TOR signaling. Rather, Myc is likely to be one of the essential factors for the control of Pol III, which only partly mediates the effects of TOR or which may function in parallel to TOR. Thus, as suggested by Reviewer 1, overexpressing dMyc alone cannot restore Pol III activity when TOR is inhibited.

2. As new data related to the downstream effectors have been added, I recommend the authors to revise the abstract to better reflect the revised content of the study.

We have now altered the abstract as suggested.
Referee #2

We thank the Reviewer for his/her support for our work and for helpful comments and suggestions on how to improve the manuscript.

Marshall et al.
Drosophila Brf is Essential for Organismal Growth and Functions Downstream of nutrient/TOR Signaling

In their revised manuscript, the authors (a) have included relevant new data concerning the roles of Maf1 and Myc in the regulation of Pol III-dependent transcription, (b) have adequately addressed most of the concerns raised by the reviewers, and (c) present their data in a much clearer way. This extended study provides significant new insights in the downstream effectors of TOR in growth regulation, which is certainly interesting to a broad readership. However, some issues need to be addressed before publication.

Specific comments

- It is questionable whether the use of Dp110 overexpression is ideal to substantiate the claim that increased TOR signaling results in an increase in Pol III-dependent transcription (Figure 4E). The authors do show that overexpression of an activated variant of S6K yield similar effects. Nevertheless, the use of UAS-Rheb (or UAS-Tsc1/2 RNAi) would be a more direct means to activate TORC1. It could be tested whether the effects of Dp110 overexpression are suppressed by reducing TOR function (genetically or by Rapamycin treatment) or in an S6K mutant background.

As suggested by the reviewer, we have now added new data showing that more direct activation of TOR signaling (using a UAS-Tsc1 RNAi line) leads to increased Pol III activity (Fig 4E). We have replaced the original figure with dp110 overexpression with this new data with tsc1 RNAi.

- The epistasis analysis shown in Figure 5 is compelling in the fat body but not in the wing imaginal disc. As previously stated, the conclusion that Brf is required for TOR-driven growth is only justified if the small clone size is not simply a consequence of apoptosis. Therefore, the experiment should be repeated in the presence of p35.

We have now repeated the experiment and examined the effects of p35 overexpression on the small clone size in both braf and braf, tsc1 mutant clones. In this case, we used en-GAL4 to both induce UAS-flp-mediated mitotic recombination and also to express UAS-p35 in the posterior compartment of the imaginal wing disc. Using this approach we confirmed our original findings that tsc1 mutant clones are overgrown, braf mutant clones are small, and braf,tsc1 clones phenocopy the single braf mutant clones and are also small. Importantly, we found that expression of p35 had no effect on the small clone size in either braf or braf, tsc1 clones (Suppl Fig 7). This data suggests that the small clone size is not simply a consequence of apoptosis.

- The new data on the role of Maf1 substantially refine the picture of how TOR acts on Pol III activity. However, all the experiments are based on a single RNAi line that is neither specified nor characterized. The authors need to show the specificity of the RNAi effect.
We generated the Maf1 RNA line in our lab by making a UAS-hairpin that targets most of the 681bp Maf1 transcript. We have added the details of how we generated the construct in the methods section. We have also included data confirming the specificity of the RNAi effect - importantly we show that the increases in tRNA synthesis caused by our UAS-Maf1 RNAi line are completely rescued by co-expression of UAS-dMaf1 (Suppl Fig 9 D, E). Also, we find that another independent UAS-Maf1 RNAi line that targets a smaller region of Maf1 has similar effects on tRNA synthesis (Suppl Fig 9 A-C). This result therefore rules out the possibility that effects we observed are simply due to the P-element insertion site of the RNAi transgenes.

- Is Maf1 phosphorylated upon high TOR activity?

We used our dMaf1 antibody to perform a western blot using both fed/starved larval lysates and control/rapamycin treated S2 cell lysates. In neither case did we see any suggestion of a mobility shift with the dMaf1 band that might indicate phosphorylation change. The question of whether Maf1 is phosphorylated is interesting. However we feel that a considerable amount of further biochemical work will need to be done to address this point adequately and would therefore be beyond the scope of this paper. For example, we could (would like to) IP Maf1 from different larval tissues and from different genotypes (gain and loss of TOR), perform mass-spec to identify potential phosphorylated residues and then generate transgenic flies carrying mutated Maf1 alleles to test the in vivo significance of the phosphorylation.

- To clarify the contribution of Myc downstream of TOR in the regulation of Pol III-dependent transcription, the authors added a series of experiments shown in Figure 7. They find that Myc overexpression under starvation conditions does not restore normal levels of tRNA(Met) expression, and they conclude that Myc is therefore unlikely to represent a link between TOR and Pol III-dependent transcription. This conclusion is problematic for several reasons:
  (1) The authors only show effects on tRNA(Met).
  (2) The activity of Myc towards Pol III-dependent transcription could be decreased upon starvation (although Myc's function in Pol II-dependent transcriptional regulation appears to be unaffected).
  (3) Starvation is not equivalent to reduced TOR activity. To be more specific, the authors should reduce TOR activity by genetic means (or by Rapamycin treatment).

We have now addressed these points. Firstly, we examined the effects of dMyc overexpression in fed/starved larvae on Pol III activity by testing additional tRNA genes as requested by the reviewer (Fig 7G). Secondly, as suggested by the reviewer, we have also performed a more specific test by using rapamycin instead of starvation (Fig 7H). In this case, the results with rapamycin were similar to, and complemented, our findings with starvation. Together these data suggest that unlike inhibition of dMaf1, maintaining high levels of dMyc is not sufficient to bypass the decrease in tRNA synthesis in either starved or rapamycin larvae. Importantly, dMyc still is able to stimulate levels of a known direct target (PPAN) under both the starvation and rapamycin conditions, hence as best as we can tell, dMyc still retains transcriptional activity under these conditions.

- Does TOR overexpression further increase the effects of Maf1 RNAi on PolIII-dependent transcription?

We examined this question by looking at the effects of expression of tsc1 RNAi and Maf1 RNAi together. We found that over activation of TOR produced a modest enhancement of the effects of Maf1 RNAi on tRNA levels (Suppl Fig 9F). This result in principle suggests that TOR may influence other events independent of Maf1 inhibition to promote tRNA synthesis. Alternatively, it could simply reflect the fact that Maf1 RNAi may not completely knockdown Maf1, hence an increase in TOR activity can act on the residual Maf1 protein to stimulate Pol III.
Reducing brf function in the fat body by means of r4-brf RNAi impacts on systemic insulin signaling but it does not decrease dIlp5 mRNA expression (Figure 2I, in contrast to the brf mutant situation shown in Figure 2H). Did the authors check other fat body drivers? Furthermore, the release of brain dILPs should be analyzed.

We carried out experiments that address both these additional points. Firstly, we expressed Brf RNAi using the cg-gal4 fat body driver (which we find gives stronger organismal growth effects than r4-gal4). In this case dILP2 and dILP5 mRNA levels were reduced (Suppl Fig 4F).

Secondly, as suggested by the reviewer, we also looked at dILP release. Leopold and colleagues showed that upon starvation, when dILP release is inhibited, dILP2 protein accumulates in the insulin-producing cells (IPC’s) in the brain (Geminard et al 2009 Cell Metab). We found that compared to control animals, r4-brf RNAi animals also showed an increased accumulation of dILP2 in the IPC’s, consistent with a reduction in dILP release. Together with our original data that measured both phospho-Akt protein and dINR mRNA levels as a readout of insulin signaling (Fig 2 and S4), these new data indicate that inhibition of Brf in the fat body leads to reduced dILP release and reduced peripheral insulin signaling; stronger inhibition of Brf (as in cg-brf RNAi or brf mutants) can additionally inhibit dILP expression levels.

Does Myc overexpression in the fat body still produce systemic growth effects when brf function is reduced?

As reported by Leopold and colleagues, we found that dMyc overexpression in the fat body (cg-brf RNAi) promotes an increase in body size. This effect was blocked when we knocked down brf by co-expression of a Brf RNAi transgene (Fig 7E). Gallant and colleagues nicely showed that normal Brf function was required for Myc-induced cell growth in the eye (Steiger et al Nat Genet 2008). Our data now extend this result to show that Brf function is required for the non-autonomous effects of dMyc on systemic growth. These data also fit nicely with our findings that normal Pol III function is required in the fat body to maintain organismal growth and body size.

Figure 1E, Figure 2B: The unit mm2 is definitively wrong. In the legend to Figure 2C, it should be indicated that the weight of 10 adult males is displayed.

We have now made the corrections. The correct unit is µm². When we converted to pdf, for some reason the font changed.

Does the title convey the key message? Non-experts will not grasp that Brf, a subunit of TFIIIB, is essential for Pol III-dependent transcription. I would suggest to emphasize that TOR regulates Pol III activity.

We struggled a little to find an apt title that is both within the 100 character (with spaces) limit, and that isn’t too verbose and cumbersome. We’re happy to take either Editor or Reviewer suggestions for alternate titles if the one we have now chosen isn’t appropriate.

General comments

The genetic analysis of brf is not very clean. The brf locus is rather complicated as it contains two other genes in an intron (lute and CG43196). The EY02964 allele is probably not a null allele and is likely to also affect lute. Furthermore, the EY insertion is an EP element that drives expression of lute in the
presence of Gal4. Did the authors test other transposon insertions in the locus? Is the severity of the phenotype enhanced when the transposon is placed over a deficiency? Of course, it is reassuring that brf RNAi yields similar results, and that the RNAi effects can be rescued by co-expression of brf. Getting the same effects with different RNAi lines (which are available) would further support the conclusions. The RNAi line used in this study should be defined.

We agree with the reviewer that the brf locus is complicated. Nevertheless, we feel we have done a good job with our loss-of-function analysis – we have used both the brf_EY02964 P-element allele and UAS-Brf RNAi to knockdown Brf. Importantly, in both cases the phenotypes we observed are completely reversed by expression of a UAS-Brf transgene (as we reported in the previous version of the manuscript). We feel that this rescue provides strong and compelling evidence that the phenotypes we observed are specifically due to loss of Brf.

We did also originally report in the previous version of the manuscript that animals transheterozygous for brf_EY02964 and a deficiency that uncovers the brf locus (Df(3R)BSC565), show the same larval growth arrest as homozygote brf_EY02964 animals. This suggests that the allele is either null or a strong hypomorph. At the early stages of this project, we originally also examined another publicly available P-element in the brf locus (brf_07161) - as now suggested by the Reviewer. We found that brf_07161 homozygous animals showed recessive lethality. However, unlike either homozygous brf_EY02964 animals or brf_EY02964/Df(3R)BSC565 animals, this lethality was at the embryonic stage and could not be reversed by ubiquitous expression of the UAS-Brf transgene. Thus while the brf_07161 may disrupt Brf function, it probably also disrupts another essential gene. Hence, we didn’t carry out any further experiments with this line. We didn’t originally report these findings in our manuscript, but we have now added them to the revised version.

Our UAS-Brf RNAi line is from the National Institute of Genetics Fly Stock Center in Japan, and we have added this information to the manuscript. As mentioned, the growth inhibitory effects we observed with this line are completely reversed by coexpression of a UAS-Brf transgene (Suppl Fig 2 F, G) indicating that the effects are specifically due to knockdown of Brf. As suggested by the Reviewer we also examined another Brf RNAi line (generated from the TRiP project and available from the Bloomington Stock Center). Unfortunately this line showed very weak knockdown of Brf and hence exhibited no obvious phenotype.

- Throughout the manuscript, the authors use the term 'TOR activity'. I guess they refer to TORC1 activity exclusively. It might be interesting to check whether TORC2 also impacts on Pol III-dependent transcription.

We do refer to TORC1 activity. We agree that it would be interesting to look at TORC2 effects, but we feel this is probably beyond the scope of the current paper.
Thank you for your patience while your manuscript has been reviewed. I apologize for the delay in responding. Your study has been sent to former referee #1, who now considers that most of his/her concerns have been properly addressed but some minor issues still remain.

As you will see below, referee #1 still suggests a number of experiments and changes for you manuscript to be accepted. As you know, it is our policy to allow for a single round of revision, but we occasionally allow for further experimental work. In this case, we agree that the interplay between TOR and myc needs to be further elucidated along the lines suggested by the reviewer before your paper can be accepted. Other points raised will not be determinant in the acceptance of your manuscript, but of course, any improvement of the final version would be in your own interest.

Do not hesitate to contact me in case you have any further questions.

Thank you again for your patience and the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely

Editor
The EMBO Journal

REFEE REPORTS

Referee #1

The further revised manuscript by Marshall and colleagues is of significantly improved quality. The authors have included important controls, e.g. for the specificity of the RNAi effects and the p35 control for the brf, Tsc1 epistasis experiment, and they have extended their analysis of dMyc's role in the regulation of Pol III-dependent transcription. The findings reported in the manuscript are interesting to a broad readership and should eventually be published. However, the story would clearly benefit from a clarification of the still quite puzzling function of dMyc downstream of TOR.

As the authors point out, the fact that dampened Pol III-dependent transcription in dMyc mutant larvae is not further reduced by Rapamycin could indicate that TOR signals via dMyc. On the other hand, the authors claim that the inability of dMyc overexpression to boost tRNA transcription upon Rapamycin treatment suggests that dMyc rather acts in parallel to TOR. However, Rapamycin probably acts on dMyc levels (and thereby activity). A role for TORC1 in stabilizing dMyc has recently been demonstrated by Bellosta and colleagues (Parisi et al., BMC Biol 2011; the authors apparently missed this publication). Consistently, the ability of overexpressed dMyc to induce the target gene ppan also appears to be reduced upon Rapamycin treatment (Figure S10, where at least a clear tendency can be seen).

Of course, the situation might be rather complex. It is conceivable that dMyc acts both downstream of TOR and independent of TOR (directly on Brf). Nevertheless, it would be valuable to further elucidate the relationship between TOR and dMyc in the regulation of Pol III transcription. For example, it might be helpful to use Tsc1/2 co-expression instead of Rapamycin to lower TORC1 activity. Does Tsc1/2 co-expression reduce tRNA levels upon dMaf1 RNAi, and does it blunt the effects of dMyc overexpression?

A critical experiment will be to test whether dMaf1 RNAi is able to restore Pol III-dependent transcription in dMyc mutant larvae.

In my previous review, I asked by mistake whether TOR overexpression would further increase the effects of dMaf1 RNAi on Pol III-dependent transcription. Of course, I wanted to ask whether dMyc overexpression would result in such an effect, and I still believe that this experiment could be telling. If dMyc functioned to inactivate dMaf1, no further increase in tRNA levels would be expected. If, on the other hand, dMyc acted independently of TOR (and directly on Brf), an additive effect should result.
Additional comments

- Figure S7 improves the epistasis analysis in mitotically active wing imaginal disc cells by inhibiting apoptosis (by means of p35 expression). The data are now much more convincing. However, I'm wondering why the authors did not apply the same technique (MARCM) as for Figures S3C-E. Inhibiting apoptosis specifically in the mutant clones would be compelling.

- Does dMyc overexpression restore the Brf levels upon brf RNAi?

- Figures 6 and S9: What are the consequences of dMaf1 overexpression? Does it result in an opposite phenotype? If so, overexpression of dMaf1 could also be used in epistasis analyses.

- A model that schematically summarizes the proposed actions of TOR and dMyc on dMaf1 and Brf to control Pol III-dependent transcription would be helpful.

Minor points

- p.8: The statement "Akt ... is activated via phosphorylation of a carboxy terminus serine residue at position 505" is not entirely correct. The main activating step appears to be the phosphorylation by PDK1, as Rictor mutants display a rather mild growth deficit despite Ser 505 not being phosphorylated.

- The inconsistent changes in dilp expression levels upon brf knockdown in the fat body are rather confusing.

- Materials and Methods:
  Both UAS-dMaf1 RNAi lines should be mentioned.
  The genotype used to achieve overexpression of dMyc should be indicated.
  The dMyc antibody is not listed.
  - Figure 2C: It should be stated in the Figure legend that the weight per 10 adult males is displayed.
  - Statistical analysis of qRT-PCR experiments: If three replicates were analyzed based on the RNA of 32 larvae each, n would be 3 (and not 32).
  - Figure S5: The results appear quite ambiguous to me. Whereas the DIC picture resembles a fat body under starvation, the Nile Red staining is more similar to a fat body under fed conditions.
  - Figure S9: It should be pointed out in the legend which RNAi line was used. Furthermore, it should be da>UAS-dMaf1 RNAi in S9F.
  - Figure S10: The color code in the second panel is wrong.
Editor Comments.

Your study has been sent to former referee #1, who now considers that most of his/her concerns have been properly addressed but some minor issues still remain.

As you will see below, referee #1 still suggests a number of experiments and changes for you manuscript to be accepted. As you know, it is our policy to allow for a single round of revision, but we occasionally allow for further experimental work. In this case, we agree that the interplay between TOR and myc needs to be further elucidated along the lines suggested by the reviewer before your paper can be accepted. Other points raised will not be determinant in the acceptance of your manuscript, but of course, any improvement of the final version would be in your own interest.

We thank the editor for their comments. It seems that the remaining issues relate to the interplay between Myc and TOR. The Reviewer states that he/she feels this interplay is “still quite puzzling”. But having read through the comments, we can’t precisely pinpoint why he/she is puzzled. The reviewer also proposes three or four new experiments in order to “further elucidate the relationship between TOR and dMyc”. Our aim with this rebuttal is to present why we think these additional experiments will not add significantly to our story - in some cases they may simply replicate results we have already presented. Instead, we feel that we can revise the Discussion to address our model, including the relationship between TOR and Myc, in more detail. We’ve incorporated the nice suggestion by the reviewer to include a model that summarizes our data, which we present below. We begin by reiterating this model and then we address the comments raised by the reviewer.

![Dietary nutrition](image)

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 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. As the Reviewer rightly points out, TOR can control Myc protein levels (Teleman et al, Parisi et al - 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. Moreover, if Myc protein levels were limiting for TOR-dependent control of Pol III, then we wouldn’t expect that knockdown of Maf1 could completely reverse the effects of rapamycin/starvation. We do find that rapamycin (TOR inhibition) doesn’t further suppress the decrease in Pol III activity in Myc null mutants. But this is probably because in the absence of Myc, Pol III activity may be approaching basal levels and can’t be significantly decreased much further. It’s important to note that we don’t completely rule out some contribution of Myc to TOR-dependent control of Pol III – it’s just that our data indicate that it is not the major contributor.

It is clear that TOR and Myc are both 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. Indeed the Reviewer makes the point that “the situation might be rather complex. It is conceivable that dMyc acts both downstream of TOR and independent of TOR”. We would suggest modifying this statement to say that dMyc might act downstream of TOR but that TOR can act independently of Myc. This “complex” relationship between Myc and TOR has been reported before: Teleman et al (2008, Cell Metab) showed using some elegant genetic approaches that over-activation of TOR signaling couldn’t promote growth when Myc was inhibited, but at the same time Myc overexpression couldn’t 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.

We can modify our discussion section to better explain all these issues and include all these points.

Reviewer Comments (in blue italics)

We are grateful to the Reviewer for his/her comments and thoughts on how to improve our paper. We appreciate the time that they have clearly taken to think about our story, and we are happy to hear that he/she thinks our work should be published.

1. The further revised manuscript by Marshall and colleagues is of significantly improved quality. The authors have included important controls, e.g. for the specificity of the RNAi effects and the p35 control for the brf, Tsc1 epistasis experiment, and they have extended their analysis of dMyc's role in the regulation of Pol III-dependent transcription. The findings reported in the manuscript are interesting to a broad readership and should eventually be published. However, the story would clearly benefit from a clarification of the still quite puzzling function of dMyc downstream of TOR.

As the authors point out, the fact that dampened Pol III-dependent transcription in dMyc mutant larvae is not further reduced by Rapamycin could indicate that TOR signals via dMyc. On the other hand, the authors claim that the inability of dMyc overexpression to boost tRNA transcription upon Rapamycin treatment suggests that dMyc rather acts in parallel to TOR. However, Rapamycin probably acts on dMyc levels (and thereby activity). A role for TORC1 in stabilizing dMyc has recently been demonstrated by Bellotta and colleagues (Parisi et al., BMC Biol 2011; the authors apparently missed this publication). Consistently, the ability of overexpressed dMyc to induce the target gene ppan also appears to be reduced upon Rapamycin treatment (Figure S10, where at least a clear tendency can be seen). Of course, the situation might be rather complex. It is conceivable that dMyc acts both downstream of TOR and independent of TOR (directly on Brf). Nevertheless, it would be valuable to further elucidate the relationship between TOR and dMyc in the regulation of Pol III transcription. For example, it might be helpful to use Tsc1/2 co-expression instead of Rapamycin to lower TORC1 activity. Does Tsc1/2 co-expression reduce tRNA levels upon dMaf1 RNAi, and does it blunt the effects of dMyc overexpression?
We used rapamycin specifically because the Reviewer suggested doing so in their initial review. (We include all of the Reviewer’s comments on Myc from the first review below):

“To clarify the contribution of Myc downstream of TOR in the regulation of Pol III-dependent transcription, the authors added a series of experiments shown in Figure 7. They find that Myc overexpression under starvation conditions does not restore normal levels of tRNA(Met) expression, and they conclude that Myc is therefore unlikely to represent a link between TOR and Pol III-dependent transcription. This conclusion is problematic for several reasons:

1) The authors only show effects on tRNA(Met).
2) The activity of Myc towards Pol III-dependent transcription could be decreased upon starvation (although Myc's function in Pol II-dependent transcriptional regulation appears to be unaffected).
3) Starvation is not equivalent to reduced TOR activity. To be more specific, the authors should reduce TOR activity by genetic means (or by Rapamycin treatment).” (Emphasis added by us)

In our revised paper we directly and fully addressed all these points. In particular, we agreed with the initial comment that starvation isn't a specific inhibitor of TOR. Therefore, based on the Reviewer's suggestion, we carried out the rapamycin expts to provide a clearer answer. Hence, we're not sure why the Reviewer is now against the use of rapamycin. He/she states that: “Rapamycin probably acts on dMyc levels (and thereby activity). A role for TORC1 in stabilizing dMyc has recently been demonstrated by Bellosta and colleagues”. But we suggest that experiments with TSC1/2 overexpression (which will also function like rapamycin to inhibit TOR) probably won't tell us anything new. Also in our Myc overexpression experiments Myc mRNA and protein levels were always well above endogenous and so it is unlikely that either Myc levels or activity were compromised in both the starved and rapamycin-fed larvae. Indeed, our data showed that in both starved and rapamycin-fed larvae, Myc overexpression still induced a known target (PPAN, Fig S10 – to help discussion we've included this figure below).
We now realize that in our resubmission we incorrectly color-coded the legend in the bottom panel of Fig S10. We have corrected this in the figure above, and we apologize for the error. This mislabeling may have led the Reviewer to interpret the data in Fig S10 to suggest that Myc effects were reduced in rapamycin-fed larvae. However, our data clearly show that the overexpressed Myc is still a strong activator in both starved and rapamycin-fed larvae (the difference in Myc activity in control and rapamycin-fed larvae - Fig S10 bottom - was minimal; in the case of control vs starved larvae - Fig S10, top - there was no difference in Myc activity). These data strongly argue that the transcriptional activity of overexpressed Myc is not affected under these conditions of reduced TOR activity.

Thus we feel the experiments with TSC1/2 (both with Myc overexpression and Maf1 RNAi expression) won’t help “elucidate the relationship between TOR and Myc” any further than our experiments with starvation and rapamycin have already. Rather we feel they will just provide another way to inhibit TOR and will therefore replicate the results we have already presented with starvation and rapamycin - its unclear to us how this will help extend our story.

2. “A critical experiment will be to test whether dMaf1 RNAi is able to restore Pol III-dependent transcription in dMyc mutant larvae.”

We show that dMyc is required for expression of Pol III factors (Fig 7A and see below) and these effects likely explain the reduced Pol III activity in dMyc mutants. We now include new data here showing that inhibition of dMaf1 (da > Maf1 RNAi), in contrast to Myc, has no effects on levels of Pol III factors (see Figure below). Hence, with respect to the proposed experiment, it is very unlikely that inhibition of Maf1 will restore Pol III-dependent transcription in Myc mutants. As such we don’t feel this experiment will extend our model or provide much more clarification of the relationship between TOR and Myc.

Figure 7

A

Left, Original Fig7A showing that Myc mutants (dm4) have reduced levels of Pol III factors.
Right, New data showing that Maf1 inhibition (da>Ma1RNAi) has no effect on levels of Pol III factors.

3. “In my previous review, I asked by mistake whether TOR overexpression would further increase the effects of dMaf1 RNAi on Pol III-dependent transcription. Of course, I wanted to ask whether dMyc overexpression would result in such an effect, and I still believe that this experiment could be telling. If dMyc
functioned to inactivate dMaf1, no further increase in tRNA levels would be expected. If, on the other hand, dMyc acted independently of TOR (and directly on Brf), an additive effect should result.”

It is unfortunate that the Reviewer made a mistake in asking us to look at TOR overactivation instead of dMyc, since we spent some time doing the expt with TOR.

Although the experiment that the Reviewer now does propose is interesting, we don’t feel the interpretation of potential results will be as clear-cut as he/she suggests. The experiment is to ask whether overexpression of Myc can enhance the elevated tRNA synthesis following Maf1 inhibition. The Reviewer suggests that, “If dMyc functioned to inactivate dMaf1, no further increase in tRNA levels would be expected”. However, we don’t propose, nor do our data suggest that dMyc inactivates Maf1. For example we find that Myc has no effects on Maf1 levels. Even if Myc did inhibit Maf1, since our RNAi-mediated knockdown of Maf1 is not complete, Myc could act to inhibit the residual Maf1 and hence an increase in tRNA levels would be seen. Hence, either potential result could be consistent with the same model. Nevertheless, the Reviewer is right in saying that it is an interesting experiment. But we feel it speaks more to how Myc functions rather than providing any more information on how TOR functions (which is the central question in our paper). Hence, we feel this experiment would be best carried out as part of a series of longer term experiments that use both genetics and biochemistry to address how Myc, Brf, Maf1 and TOR interact and cooperate genetically and biochemically to control Pol III (e.g. Does TOR phosphorylate any of these factors? What is the dynamics of biochemical interactions between these factors and their association at Pol III genes? How does phosphorylation alter these interactions? What are the consequences of genetically altering putative phosphorylation sites?). These are certainly avenues that we intend to explore in the future.

Additional comments

- Figure S7 improves the epistasis analysis in mitotically active wing imaginal disc cells by inhibiting apoptosis (by means of p35 expression). The data are now much more convincing. However, I'm wondering why the authors did not apply the same technique (MARCM) as for Figures S3C-E. Inhibiting apoptosis specifically in the mutant clones would be compelling.

We feel that the approach we used provides a clear result: brf or brf,tsc1 mutant clones are not small simply due to cell death. We used this approach because we had the stocks ready to go in order to get the expts done within the revision timeframe.

- Does dMyc overexpression restore the Brf levels upon brf RNAi?

Brf levels are not restored

- Figures 6 and S9: What are the consequences of dMaf1 overexpression? Does it result in an opposite phenotype? If so, overexpression of dMaf1 could also be used in epistasis analyses.

Maf1 overexpression does not have any effect on Pol III activity

- A model that schematically summarizes the proposed actions of TOR and dMyc on dMaf1 and Brf to control Pol III-dependent transcription would be helpful.

We can add a model if it helps

Minor points
- p.8: The statement "Akt ... is activated via phosphorylation of a carboxy terminus serine residue at position 505" is not entirely correct. The main activating step appears to be the phosphorylation by PDK1, as Rictor mutants display a rather mild growth deficit despite Ser 505 not being phosphorylated. We will correct the text

- The inconsistent changes in dilp expression levels upon brf knockdown in the fat body are rather confusing.

We interpret this result to mean that strong knockdown of Brf can suppress fat-brain signaling enough to reduce both dilp expression and release, whereas a weaker knockdown may influence dILP release alone.

- Materials and Methods:

  - Both UAS-dMaf1 RNAi lines should be mentioned. We will correct the text

  - The genotype used to achieve overexpression of dMyc should be indicated. We will correct the text

  - The dMyc antibody is not listed. We will correct the text

- Figure 2C: It should be stated in the Figure legend that the weight per 10 adult males is displayed. In the Materials and Methods section, the authors claim that "average weights per adult are presented here", which is not the case. We will correct the text

- Statistical analysis of qRT-PCR experiments: If three replicates were analyzed based on the RNA of 32 larvae each, n would be 3 (and not 32). We will correct the text

- Figure S3: It should be UAS-p35 in the legend. We will correct the text

- Figure S5: The results appear quite ambiguous to me. Whereas the DIC picture resembles a fat body under starvation, the Nile Red staining is more similar to a fat body under fed conditions.

  We feel the nile red staining, like the DIC image, shows large lipid droplets typical of a starved fat body.

- Figure S9: It should be pointed out in the legend which RNAi line was used. Furthermore, it should be da>UAS-dMaf1 RNAi in S9F.
We will correct the text

*Figure S10: The color code in the second panel is wrong.*

We will correct the text
I have contacted both referees and I am glad to tell you that they agree that your manuscript is ready for publication. As you will see below, referee #1 still proposes one experiment that, in his opinion, would strengthen your views on the relationships among TOR, myc and Maf1, but s/he explicitly remarks that it would not be necessary for acceptance. If you have already this piece of information or you think the experiment can be performed in a timely manner, I would recommend you to include it, although it will not be considered necessary for acceptance.

Once you have included the referee corrections and clarifications suggested here and in previous reports, you will receive an official acceptance letter with further instruction on how to proceed with the publication process.

I look forward to seeing the final version of your manuscript. Do not hesitate to contact me in case you have any questions.

Thank you very much for your contribution to The EMBO Journal.

Yours sincerely

Editor
The EMBO Journal

REFEE REPORT

Referee #1

As pointed out in my previous reviews, the data presented by Marshall and colleagues are new and exciting. The authors have included an astonishing amount of results, illuminating many aspects of TOR-dependent growth regulation via Pol III-dependent transcription. In my opinion, these findings clearly merit publication in EMBO Journal.

The model that the authors now propose is very helpful and should definitively be included in the final version. However, I am convinced that it would be in the authors' best interest to clarify whether the main inhibitory sign from TOR (I would rather call it TORC1) to Maf1 is (in part) mediated via Myc. This question remains largely unanswered, and most of my suggestions were aiming at solving this issue. In my view, it is in the responsibility of the authors whether they want to include these further experiments.

Personally, I would tackle at least one additional test. To exclude that dMyc's major role downstream of TORC1 is to control the inhibition of dMaf1, the effects of dMaf1 RNAi in dMyc mutants could be assessed. If, as the authors assume, dMaf1 RNAi did not restore Pol III-dependent transcription, it could be concluded that Myc does not primarily act upstream of dMaf1. Such a finding would clearly substantiate the model. Thus, I would recommend performing this experiment.

The additional data demonstrating that dMaf1 RNAi does not impact on the levels of Pol III factors should be included in Figure 7. However, they should rather be compared with dMyc overexpression (as dMaf1 RNAi results in an increase in Pol III activity).

Minor comments:
1) Maf1 overexpression does not have any effect on Pol III activity. These data should be mentioned (either in the main text or in the figure legend).
2) Differential dilp regulation by brf RNAi:
We interpret this result to mean that strong knockdown of Brf can suppress fat-brain signaling enough to reduce both dilp expression and release, whereas a weaker knockdown may influence dILP release alone.
The inconsistency I was referring to lies in the transcriptional regulation of the dilp
genes. Whereas only dilp5 transcription is reduced in brf mutants (Figure S4G), the downregulation of brf by means of cg>brf RNAi reduces dilp5 and dilp2 transcription (Figure S4F). In contrast, it has been reported by several groups that the transcription of dilp2 remains unchanged upon starvation.

2nd Revision - authors' response 23 January 2012

We have addressed all the final Reviewer concerns and issues in full. In particular, we have expanded our Discussion section to include a more detailed discussion of what our data tells us about the relationship between TOR, Myc and Maf1 in the control of Pol III transcription (see paragraphs 4 and 5 is Discussion section). As suggested by the reviewer, we have also added a model figure (Fig 8). We have also included our new data showing that knockdown of Maf1 has no effect on the mRNA levels of Pol III factors (unlike Myc). The Reviewer suggested including this data in Fig 7. But we felt that this Figure was already quite dense. Instead we felt a more appropriate action would be to include the result in Figure S9, along with some more new data showing that Maf1 knockdown doesn’t influence either pre-rRNA (Pol I-dependent) or rp49 mRNA (Pol II-dependent) – again unlike Myc. These data further underscore how Myc and Maf1 function differently. We have discussed this new data in both the Results and Discussion section (p.11/12 and p.16). Finally, we have made all the various minor changes and corrections to the text and figures suggested by the reviewer.