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TOR and S6K1 promote translation reinitiation of uORF-containing mRNAs via phosphorylation of eIF3h

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(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.)

Editor: Anne Nielsen

1st Editorial Decision 12 October 2012

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see, while referees 2 and 3 express some interest in the work and topic in principle, they raise a number of technical concerns and requests for additional control experiments. At the same time referee 1 is rather negative and finds that the experimental data provided fails to support the overall conclusions of the manuscript. I will not repeat all the individual points of criticism here, but many points are shared between the referees, and it becomes clear that the depth of analysis is too limited and that the study is thus too premature for the referees to support its publication in The EMBO Journal.
Clearly, an extensive amount of further experimentation would be required to clearly establish that TOR works through S6K1 to promote translation re-initiation and to bring the study to the level of insight and significance required for publication here. Furthermore, the outcome of such additional experiments cannot be predicted at this point, and would thus lie outside the scope and timeframe of a revision. I therefore see little choice but to come to the conclusion that we cannot offer to publish the manuscript at this point. We could potentially be willing to consider a completely new submission of the manuscript at a later stage, but only in the case where all criticisms raised have

been addressed to the full satisfaction of all referees, especially referee #1.

Thank you in any case for the opportunity to consider this manuscript. I am sorry we cannot be more positive on this occasion, but we hope nevertheless that you will find our referees' comments helpful.

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This study concerns the mechanisms involved in the reinitiation of translation of mRNAs in plant cells. Many plant mRNAs contain upstream open-reading frames which necessitate translation of the main protein-coding open-reading frame by this mechanism. The present authors follow up on their earlier studies by examining the role of phosphorylation of eIF3h, one component of the multisubunit factor eIF3, in promoting reinitiation.

The authors interpret their data as indicating that signaling through the target of rapamycin, TOR, promotes phosphorylation of eIF3h and the reinitiation of translation.

There are a considerable number of concerns with the data, their interpretation and their interpretation.

Major points:

1. Fig. 2: how do the authors know that the mRNAs they studied are actually expressed in the forms indicated, i.e., with the uORF-containing 5'-UTRs, rather than in shorter forms from alternative promoters? It is also surprising that, if 30% of Arabidopsis mRNAs contain uORFs (Zhou et al., 2010), Torin1 has essentially no effect on the overall ribosomal profile 9 the numbers of polysomes are the same +/- Torin1. What is the explanation for this?

2. P. 8, lines 19-20: I do not understand what logic leads to the statement that 'Torin-1 did not affect the first initiation event'. Torin-1 markedly decreases the proportion of all the uORF-mRNAs found in polysomes; some remain associated with small polysomes, but this is exactly what one would expect if initiation onto these mRNAs is inhibited - less frequent initiation = fewer, smaller polysomes.

3. P. 9, lines 4-7: where are the data for phosphorylation of TOR? They are not in Fig. S2C are the authors referring to a panel of Fig. 2, perhaps?). In fact, the S2448 is not conserved at all in Arabidopsis TOR, so it is not clear what any such data would mean in any case. They are certainly not reporting TOR signaling in a similar way to p-S2448 in mammalian TOR. What is this antibody actually detecting?

4. Fig. 2E and elsewhere (e.g., Figs. 3 and 4); the authors need to show that the fraction at the right-hand end of the blot is indeed 40S subunits rather than cytoplasmic material at the top of the gradient. For example, in Fig. 2E, top and middle, the strongest signals for S6K1 and TOR are in fractions that contain little or no rRNA. These are obviously not ribosomal fractions. The blot for TOR from the Torin1 sample (bottom part of Fig. 2E) is much weaker than the others making it hard to discern the distribution of TOR. Related to the same figure, the authors need to be sure that the concentrations of Torin1 they use are fully effective, otherwise it is hard to interpret the data.

5. Fig. 3B vs Fig. 2B; why are there substantial changes in some mRNAs in Fig. 3B but not Fig. 2B?

6. I was unable to find data showing the effectiveness of the RNAi for TOR - i.e., blots from control and RNAi cells.

7. Fig. 3C: does Torin1 affect total TOR levels? It is not clear why TOR id detected in NAA or Mock samples, but not in the Torin1 set.

8. Fig. 4C: it is crucial to show data that allow a direct comparison between the S6K1-P and S6K1 levels in control and RNAi-treated cells, rather than supplying them as part of separate figures.

9. Fig. 5B: as drawn, this image is not helpful.

10. Fig. 5C: NAA causes eIF3h to shift to the right (basic side) on the 2D gel. This is completely inconsistent with an increase in phosphorylation which would clearly make the protein more acidic. The observed changed in behaviour cannot be due to phosphorylation.

11. P. 13, line 10: it is an overinterpretation to state the data 'indicate' a role for eIF3h phosphorylation in its association with polysomes. Torin1 may cause its dissociation for other reasons. Next line: 'partially phosphorylated' - phosphorylation, especially as revealed by a P-specific antibody, is strictly quantized - either the protein is phosphorylated or it isn't; it cannot be 'partially phosphorylated'.

12. Fig. 7C: it is not clear to this reviewer that the authors have shown the effect of eIF3h and the S178D mutant are specific to uORF-containing mRNAs. Do they also affect other mRNAs, e.g., pmonoGUS?

13. Fig. 8: Torin1 inhibits all known functions of TOR complexes 1 and 2, and these complexes play multiple and diverse roles in cell physiology, including control of anabolic metabolism, autophagy, cell growth, cell division and ribosome biogenesis. Thus, although the data in Fig. 8 are interesting, it is a big stretch to link them specifically with effects on eIF3h. There may be many other factors involved.

14. Fig. 8D: it is not at all clear how phosphorylation of eIF3h would aid reinitiation. While a detailed analysis of the mechanism would clearly require substantial additional work, the authors should at least propose possible mechanisms.

Other points:

1. Fig. S1A; what do the times indicate? Time of treatment of plants with Torin1? Or of cell cultures?

2. P.7, lines 5-3 up: are there actually any proven examples of plant mRNAs that contain a functional 5'-TOP? It is generally thought that such mRNAs are restricted to higher metazoan organisms.

3. P. 11, line 17: the data referred to do not seem to be in Fig. S1A.

4. Fig. 7A: the labeling seems to be wrong - I assume both constructs were used in each panel.

5. It would be help the reviewers if the authors would number the actual figures.

6. It is not clear why the authors refer to S6K1 - are there multiple isoforms in plants, as in mammals? If so, how do the authors know the effects they see are due to this isoform? If not, why do they term this protein S6K1 (not S6K)?

Referee #2:

The studies by Schepetilnikov et al describe the role of Arabidopsis TOR in reinitiation after upstream open reading frame (uORF) translation. They found that the phytohormone auxin can promote phosphorylation of TOR and S6K1, polysome localization of TOR, and enhanced loading of uORF mRNAs into polysomes. TOR inhibition using Torin1 or depletion by RNAi blocked these effects of auxin. On the other hand, mutation of the eIF3 subunit eIF3h only abolished polysome loading of the uORF mRNAs but not TOR localization to the polysomes, suggesting that eIF3 could be acting downstream of TOR. They further demonstrated that eIF3h is phosphorylated in an auxin-responsive and Torin1-sensitive manner and that phosphorylated eIF3h is enriched in the polysomes.

Using reporter assays, they verified that TOR inhibition or partial depletion and eIF3h mutation could diminish the activity of a reporter that undergoes reinitiation. Finally, they correlated this auxin-responsive function of TOR with root gravitropic responses.

Overall, the studies are interesting and the data are consistent with TOR promoting translation of uORF-containing mRNAs and that eIF3h is phosphorylated in a TOR-dependent manner. The data on that supports that it is the reinitiation that is TOR-dependent could be strengthened. The following are some specific comments:

1. In Figure 2A, figure legend should describe what the cartoon depicts (are the boxes representing the number of uORFs?).

2. Figure 2B demonstrates that uORF-containing mRNAs are being actively translated (found in the polysomes). The pattern differs however among some of these mRNAs (ie amounts in the different polysome gradients). Does this correlate with the amount of uORFs?

3. In Figure 3C, why is S6K more abundant in the non-polysome fraction of TOR-depleted (mock-treated) strain? Decreased TOR activity should promote S6K binding to polysomes according to the model.

4. According to the model, S6K regulates eIF3h. The authors should examine if the phosphorylation of eIF3h at S178 is mediated by S6K. Binding of eIF3 mutants to S6K and TOR should also be examined to support their model.

5. Figures 6 and 7 are important as they more directly address how TOR is involved in translation reinitiation. First of all, the authors should describe in the text what MAGRIS is. It was not clear to this reviewer why the efficiency of initiation (GUS/GFP activity) was similar between pmonoGUS and pMAGRIS-GUS. As it appears that the presence of 2 uORFs (in ARF5) can decrease translation efficiency, analysis of other uORFs presented in Fig 2 and the effect of deletion/mutation of uORFs for some of this (eg ARF5) would help support the main conclusion of the study that TOR plays a role in translation reinitiation. Would having more uORF be more sensitive to TOR depletion?

Minor comment
Several phrases use "Torin1-dependent", this should be rephrased to Torin1-sensitive for clarity.

Referee #3:

The manuscript titled, "TOR and S6K1 promote translation reinitiation of uORF-containing mRNAs via phosphorylation of eIF3h" by Schepetilnikov et al. describes a molecular connection between TOR and reinitiation after upstream open reading frame translation (uORF) in plants.

This study reveals that treatment of Arabidopsis with 1-naphthylacetic acid (NAA- an analogue of the phytohormone auxin) results in activation of the TOR pathway, and binding of TOR to the noncore subunit of the eIF3 complex, eIF3c, while S6K1 interacts with this subunit only in conditions of TOR inhibition with Torin1. Previously, a similar mechanism was elegantly described in mammals in response to growth factor/hormone stimulation. Schepetilnikov et al., however, further demonstrate that TOR activation results in increased polysomal loading of uORF-containing mRNAs, specifically Auxin responsive factors (ARFs) mRNAs, while TOR inhibition with Torin1 shifted from polysomes to the 40S fraction. Interestingly, the authors identified eIF3h as a potential TOR downstream effector involved in the translation of these uORF-mRNAs. Finally, this study shows that depletion of TOR in plants results in alterations in root gravitropic responses, demonstrating a role for TOR in plant development.

While overall the paper is interesting and innovative, the authors need to answer several questions and strengthen the manuscript in a few areas before publication in the EMBO Journal can be recommended.

Comments:

1- This study uses NAA and Torin1 as tools to modulate TOR activity in Arabidopsis. The authors need to further evaluate the effects of NAA on other signaling pathways, including the MAPK pathway, in their model.

Also, experiments in which pre-treatment of seedlings with Torin1 followed by NAA stimulation will be necessary to further demonstrate that the effects of NAA on polysomal loading of uORF-mRNAs (Fig. 2B) occurs specifically via activation of TOR signaling.

Also, since some S6K1-phosphorylation is observed in basal conditions (Fig 1B), we would expect to detect TOR associated with both the polysomes and the 40S fraction in the WT/mock panel of Fig. 2E. Furthermore, this figure will require better blots of both S6K1 P-T449 and P-TOR.

2- Figure 3 demonstrates that the knockdown of TOR in Arabidopsis decreases the association of ARFs to polysome. What is the knockdown efficiency for TOR RNAi shown in Figure 3? The authors need to show that a control RNAi seedling shows similar results as the ones presented in Figure 2.

3- The knockdown of S6K1 or the rescue with expression of a constitutively active mutant of S6K1 in TOR-depleted seedlings (Figure 3) would add strength to the role of TOR/S6K1 on the polysomal loading of uORF-mRNAs.

4- Figure 4 and 5 suggest that eIF3h functions downstream of TOR and S6K1 in promoting reinitiation of uORF-mRNAs. Another initiation factor, eIF3b, has been shown to be a target of S6K1 involved in cap-dependent translation in mammals. Is the knockdown of eIF3b able to impair NAA-induced uORF-mRNAs reinitiation?

5- The authors need to further characterize eIF3h as a potential target of S6K1. Co-immunoprecipitation experiments of S6K1-eIF3h and TOR-eIF3h are missing, and it would be interesting to assess the binding dynamics in response to NAA treatment and in Torin1-pretreated seedlings. In vitro kinase assay showing that S6K1 can phosphorylated the putative RXRXXS site under conditions of NAA will be suitable. As for S6K1, does the expression of eIF3h S178D in TOR-depleted or Torin1-treated seedlings rescue the decrease in polysomes loading?

6- What is the effect of eIF3h S178A and S178D on root gravitropism?

Resubmission 07 January 2013

Reviewer 1

Major points:

I hope that we have addressed the referee #1 concerns by adding new data and/or modifying the text.

"1. Fig. 2: how do the authors know that the mRNAs they studied are actually expressed in the forms indicated, i.e., with the uORF-containing 5'-UTRs, rather than in shorter forms from alternative promoters?"

RNA samples were reverse transcribed into cDNA using reverse transcriptase with oligo-$(dT)_{18}$ primer. To control the presence of the intact 5'-leader for each cDNA we have performed semi-quantitative RT-PCR with the pair of gene-specific primers to the 5'-leader and the corresponding coding region. The length of the obtained fragment was compared with DNA markers. To our knowledge, alternative promoters for selected mRNAs which transcription are under intensive studies in plants were not reported.

"It is also surprising that, if 30% of Arabidopsis mRNAs contain uORFs (Zhou et al., 2010), Torin1 has essentially no effect on the overall ribosomal profile 9 the numbers of polysomes are the same +/- Torin1. What is the explanation for this?"

According several reports, in plants about 20% of mRNAs were predicted to be loaded with mostly one uORF (often reinitiation permissive if enough short), and about 10%—with two and more ORFs, and about 5%—contain the most inhibiting uORFs that are longer than 30 codons. In many cases uORF locates close to the 5'-end of mRNA, or the start codon initiation context is not favourable and their recognition is questionable, and thus can be bypassed by leaky scanning. *Trans*factors and *cis*RNA elements may impede or stimulate reinitiation as well.

Now we present direct superimposition of selected polysomal profiles as supplemental data (Fig. S5B) obtained from seedlings treated by NAA or Torin-1. Small, but significant decrease in WT heavy polysomes and in *TOR RNAi* polysomal levels was detected in response to Torin-1 application. Interesting that heavy polysomes detected in WT plants are less pronounce in *TOR RNAi* or *eif3h-1* mutant plants. One might propose that at some extend a new ribosome entry at uORF-mRNA was abolished due to pause of reinitiating ribosomes at uORF or main ORF.

«*2. P. 8, lines 19-20: I do not understand what logic leads to the statement that 'Torin-1 did not affect the first initiation event'. Torin-1 markedly decreases the proportion of all the uORF-mRNAs found in polysomes; some remain associated with small polysomes, but this is exactly what one would expect if initiation onto these mRNAs is inhibited - less frequent initiation = fewer, smaller polysomes.*»

First of all, loading of uORF-less mRNAs on heavy polysomes is not significantly affected by Torin-1 or in our mutants. "Smaller or fewer" polysomes are seen for loading of uORF-mRNAs in WT plants treated by Torin-1, or in *TOR RNAi* and eif3h-1 seedlings. The possible explanation is that initiation at the second or further downstream uORF or main ORF was impeded. Here, uORF-mRNA is still 'polysomal', but mainly bound by 1-2 ribosomes.

"3. P. 9, lines 4-7: where are the data for phosphorylation of TOR? They are not in Fig. S2C are the authors referring to a panel of Fig. 2, perhaps?)."

TOR phosphorylation data were presented before in Fig. 2D (panels 3, 4). Now these are presented in Fig. 2E (panels 3, 4—TOR-P). We now include addition citation for Fig. 2E in the text.

"In fact, the S2448 is not conserved at all in Arabidopsis TOR, so it is not clear what any such data would mean in any case. They are certainly not reporting TOR signaling in a similar way to p-S2448 in mammalian TOR. What is this antibody actually detecting?"

Alignment of TOR sequences from *human* (EAW71683), *rattus norvegicus* (NP_063971), *Arabidopsis* (NP_175425), *Zea mays (*NP_001105293) and *Oryza sativa* (Q0DJS1) showing conservation of S2448 is included into Fig. 2 as Fig. 2D.

« *4. Fig. 2E and elsewhere (e.g., Figs. 3 and 4); the authors need to show that the fraction at the right-hand end of the blot is indeed 40S subunits rather than cytoplasmic material at the top of the gradient. For example, in Fig. 2E, top and middle, the strongest signals for S6K1 and TOR are in fractions that contain little or no rRNA. These are obviously not ribosomal fractions."*

In polysomal profiles, monosomes and ribosomal subunits are not well separated by sucrose

gradient sedimentation. Although 28S rRNA is readily detected, 18S rRNA is often detected barely on agarose gels. 60S/40S migrate in fractions 7 and 8 (their profiles indicated) from 11 total sucrose gradient fractions. Accordingly, 40S-containing preinitiation complexes normally sediment in fraction 8 and attract max of mRNA in response to Torin-1.

However, we repeated experiments earlier presented as Figs. 2E (WT/ Mock) and 3C (*TOR RNAi/* Mock), and now these are included as Figs. 3A (WT/ Mock) and 4D (*TOR RNAi*/ Mock). In addition, intensity of other rRNA images was increased.

"The blot for TOR from the Torin1 sample (bottom part of Fig. 2E) is much weaker than the others making it hard to discern the distribution of TOR. Related to the same figure, the authors need to be sure that the concentrations of Torin1 they use are fully effective, otherwise it is hard to interpret the data.»

We propose that TOR associates with polysomes when is phosphorylated. Torin-1 inactivates TOR and triggers its dissociation from polysomes. Thus, inactive S6K1 and not TOR mainly associates with polysomes in Torin-1-treated seedlings. But some TOR-P is seen in 40S-containing fractions. The Fig. 2E is now presented as Fig. 3C.

Honestly, we are very lucky that standard concentration of Torin-1 (250 nmol) used to treat seedlings is sufficient to block reinitiation without significantly affecting initiation events. Further work is planned to study TOR effects on cap-dependent initiation of translation in plants.

« 5. Fig. 3B vs Fig. 2B; why are there substantial changes in some mRNAs in Fig. 3B but not Fig. 2B?»

Transcription of at least some ARF-encoded mRNAs, but not bZIP11 mRNA, is somewhat triggered by auxin transiently in a manner sensitive to TOR levels. See Fig. S4B-E for details. This has now been clearly explained in the text (page 11, the second para). Figs 2B and 3B are presented now as Figs 2C and 4B.

« 6. I was unable to find data showing the effectiveness of the RNAi for TOR - i.e., blots from control and RNAi cells.»

Western blot analysis of WT and *TOR RNAi* seedlings are presented now as Fig. 4C. Additional western blot analysis for WT and *eif3h-1* seedlings are presented as Fig. 5C.

«7. Fig. 3C: does Torin1 affect total TOR levels? It is not clear why TOR id detected in NAA or Mock samples, but not in the Torin1 set.»

Fig. 3C is presented now as Fig. 4D. Torin-1 does not affect total TOR levels (see Fig. S2A in Schepetilnikov et al., 2011). In TOR-deficient plants, TOR seems to be dephosphorylated in the presence of Torin-1, and found neither in polysomes, no in 40S-containing fractions. Inactive S6K1 binds polysomes/ribosomal subunits instead.

"8. *Fig. 4C: it is crucial to show data that allow a direct comparison between the S6K1-P and S6K1 levels in control and RNAi-treated cells, rather than supplying them as part of separate figures."*

Fig. 4C is now presented as Fig. 5D. Due to limited space it is difficult to combine western blots for WT, *TOR RNAi* and *eif3h-1* as one Fig. We included now direct comparison between the S6K1-P and S6K1 (eIF3h and eIF3h-P) levels in WT and TOR RNAi extracts (Fig. 4C) as well as in WT and

eif3h-1 extracts as Fig. 5C.

"9. Fig. 5B: as drawn, this image is not helpful."

The 3D image was turned around to show clearly accessibility of S178 within eIF3h.

"10. Fig. 5C: NAA causes eIF3h to shift to the right (basic side) on the 2D gel. This is completely inconsistent with an increase in phosphorylation which would clearly make the protein more acidic. The observed changed in behaviour cannot be due to phosphorylation.»

We are especially grateful to this reviewer for pointing out our mistake. We apologize for inverting basic and acidic proximities of the 2D gel. To convince you, we repeated the experiment twice using different strips. One of those gels is included now as Fig. 6C.

"13, line 10: it is an overinterpretation to state the data 'indicate' a role for eIF3h phosphorylation in its association with polysomes. Torin1 may cause its dissociation for other reasons. Next line: 'partially phosphorylated' - phosphorylation, especially as revealed by a P-specific antibody, is strictly quantized - either the protein is phosphorylated or it isn't; it cannot be 'partially phosphorylated'."

The statement was removed from the text; 'partially phosphorylated was replaced by phosphorylated (page 13, line 4 up).

"12. Fig. 7C: it is not clear to this reviewer that the authors have shown the effect of eIF3h and the S178D mutant are specific to uORF-containing mRNAs. Do they also affect other mRNAs, e.g., pmonoGUS?"

Fig. 7C is presented as Fig. 8C now. The required control is included in Fig. 8C.

"13. Fig. 8: Torin1 inhibits all known functions of TOR complexes 1 and 2, and these complexes play multiple and diverse roles in cell physiology, including control of anabolic metabolism, autophagy, cell growth, cell division and ribosome biogenesis. Thus, although the data in Fig. 8 are interesting, it is a big stretch to link them specifically with effects on eIF3h. There may be many other factors involved."

Fig. 8 is now presented as Fig. 9. We agree with the reviewer. However, we do not claim to do so, and this was not the aim of the experiment. We like to strengthen that there is a correlation between the auxin-responsive functions of TOR that are sensitive to Torin-1 with root gravitropic responses.

«14. Fig. 8D: it is not at all clear how phosphorylation of eIF3h would aid reinitiation. While a detailed analysis of the mechanism would clearly require substantial additional work, the authors should at least propose possible mechanisms.»

The results indicate that eIF3h phosphorylation possibly alters the binding affinity of eIF3h to polysomes. It seems unlikely that eIF3h functions at the essential initiation step. We propose a role for eIF3h-P in recruitment of eIF3 to polysomes. Discussion on possible eIF3h role in reinitiation is now presented (page 21, the last para).

«Other points:

1. Fig. S1A; what do the times indicate? Time of treatment of plants with Torin1? Or of cell cultures?»

Times of recombinant S6K1 incubation with extracts prepared from WT or *TOR RNAi* plants with or without Torin-1. The Fig legend was improved.

"2. P.7, lines 5-3 up: are there actually any proven examples of plant mRNAs that contain a functional 5'-TOP? It is generally thought that such mRNAs are restricted to higher metazoan organisms."

Many mRNAs in *Arabidopsis*, including those coding for ribosomal proteins, are loaded with TOP or TOP-like motifs within the leader regions. Although there are data showing that (r)-protein mRNAs that harbour 5' TOP-like sequences are differentially up-regulated during germination, the role of TOR in their translation was not investigated.

"3. P. 11, line 17: the data referred to do not seem to be in Fig. S1A."

Page 11, line 1 up. The data are presented in Fig. S1A as a third (right) panel (*TOR RNAi/* Mock). The recombinant S6K1 was not phosphorylated by extracts from *TOR RNAi* seedlings.

"4. Fig. 7A: the labeling seems to be wrong - I assume both constructs were used in each panel."

The labelling was corrected.

5. It would be help the reviewers if the authors would number the actual figures.

According suggestion of this reviewer we numbered our figures to 9.

6. It is not clear why the authors refer to S6K1 - are there multiple isoforms in plants, as in mammals? If so, how do the authors know the effects they see are due to this isoform? If not, why do they term this protein S6K1 (not S6K)?

The *Arabidopsis* genome encodes two S6K1 homologs, S6K1 (p70) and S6K2 (p85), where S6K1 is phosphorylated by TOR at the hydrophobic motif residue T449 (Zhang et al, 1994; Schepetilnikov et al, 2011). Both are labeled by anti S6K1 antibodies (see, for example Fig. 4C), however in our conditions only S6K1 is phosphorylated in response to auxin.

Reviewer 2

Reviewer 2 concluded that our studies are interesting and the data are consistent.

"The following are some specific comments:

1. In Figure 2A, figure legend should describe what the cartoon depicts (are the boxes representing the number of uORFs?)."

The description was included in the Fig. 2A legend.

"2. Figure 2B demonstrates that uORF-containing mRNAs are being actively translated (found in the polysomes). The pattern differs however among some of these mRNAs (ie amounts in the different polysome gradients). Does this correlate with the amount of uORFs?"

It might correlate with the amount of translated uORFs. Although the loading of the ARF6 leader is less efficient that of ARF5, or ARF 11, these three leaders contain 6 uORFs.

"3. In Figure 3C, why is S6K more abundant in the non-polysome fraction of TOR-depleted (mock-treated) strain? Decreased TOR activity should promote S6K binding to polysomes according to the model."

The experiment with TOR-depleted (mock-treated) seedlings was repeated, and now presented in Fig. 4D (upper panel). However, inactive S6K1 accumulation in polysomes seems to be less efficient than in ribosome and subunit fractions. One might assume that S6K1 phosphorylation (and thus inactive S6K1 binding) by TOR within eIF3-containing PIC is a first priority. It explains why reinitiation is limited in eukaryotes.

"4. According to the model, S6K regulates eIF3h. The authors should examine if the phosphorylation of eIF3h at S178 is mediated by S6K. Binding of eIF3 mutants to S6K and TOR should also be examined to support their model."

First, we compared phosphorylation levels of S6K1 and eIF3h in WT and *TOR RNAi* extracts (Fig. 4C). No significant amount of phosphothreonine 449 and phosphorylated eIF3h were detected in TOR-deficient seedlings with our antibodies, further suggesting that S6K1 or other TOR-dependent kinase are major effectors of eIF3h phosphorylation.

Additionally, data on complex formation between eIF3h and S6K1, and/or TOR are presented as new Fig. 6H. Direct interaction between eIF3h and S6K1 was demonstrated by yeast two-hybrid system and now shown as new Fig. S6.

"5. Figures 6 and 7 are important as they more directly address how TOR is involved in translation reinitiation. First of all, the authors should describe in the text what MAGRIS is. It was not clear to this reviewer why the efficiency of initiation (GUS/GFP activity) was similar between pmonoGUS and pMAGRIS-GUS.

Figs 6, 7 are presented now as Figs 7, 8. An artificial uORF that encodes polypeptide MAGRIS (uORF MAGRIS) is now described well in the text. Many single uORFs, including a 7-codon uORF MAGRIS, are reinitiation permissive in mock seedlings and, when translated, do not impede downstream main ORF recognition and translation. Our data suggest that inhibition can be revealed as a result of TOR inactivation by Torin-1.

"As it appears that the presence of 2 uORFs (in ARF5) can decrease translation efficiency, analysis of other uORFs presented in Fig 2 and the effect of deletion/mutation of uORFs for some of this (eg ARF5) would help support the main conclusion of the study that TOR plays a role in translation reinitiation.

Yes, the ARF3 leader harbours two uORFs. As this reviewer suggested, we replaced both uORF start codons by stop codons to remove uORFs1 or 2, or both together. The results of the transient

expression of ARF3-leader containing reporter mRNAs are presented as Fig. 7C and D.

Would having more uORF be more sensitive to TOR depletion?"

ARF11 seems to be less sensitive to TOR depletion, but its polysomal loading is affected as well. Our data indicate that reinitiation after translation of 2-3 uORFs is more sensitive to TOR inactivation than single uORF (Fig. 7). Often some uORFs are bypassed, if TC was not recruited. It was demonstrated for *ARF5*, where some uORFs are bypassed by ribosomes and thus are dispensable for main ORF translation (Roy et al., 2010).

"Minor comment

Several phrases use "Torin1-dependent", this should be rephrased to Torin1-sensitive for clarity."

The reviewer's suggestion was adopted

Reviewer 3

"1- This study uses NAA and Torin1 as tools to modulate TOR activity in Arabidopsis. The authors need to further evaluate the effects of NAA on other signaling pathways, including the MAPK pathway, in their model."

In plants, pathways leading to TOR activation are not known. But according to this reviewer suggestion, we have added an additional experiment to compare phosphorylation kinetics of TOR and three *Arabidopsis* ERK-1/2-like MAPKs in response to auxin. The results are presented as the panel 3 in Fig. S3C, and indicate no correlation between phosphorylation of TOR and MAPKs during long term of auxin application.

"Also, experiments in which pre-treatment of seedlings with Torin1 followed by NAA stimulation will be necessary to further demonstrate that the effects of NAA on polysomal loading of uORF-mRNAs (Fig. 2B) occurs specifically via activation of TOR signaling."

The experiment is presented now as Fig. S3A, B. The main result is that inactivation of TOR by Torin-1 diminished auxin effects on loading of uORF-mRNAs.

"Also, since some S6K1-phosphorylation is observed in basal conditions (Fig 1B), we would expect to detect TOR associated with both the polysomes and the 40S fraction in the WT/mock panel of Fig. 2E. Furthermore, this figure will require better blots of both S6K1 P-T449 and P-TOR."

The experiment shown in Fig. 2E (WT/ mock panel) has been repeated and is presented now as Fig. 3A. However, inactive S6K1 preferentially associates with polysomes and fractions of ribosome/subunits. TOR was mainly detected in fractions of 80S/60S/40S, but co-sediments with polysomes as well. However, in polysomes TOR-P was at the limit of detection of our phospho-antibodies. Thus, TOR activation is limited in mock plants. And TOR/S6K1 loading on 40S PIC is a first priority possibly to support the cap-dependent mechanism of translation initiation.

"2- Figure 3 demonstrates that the knockdown of TOR in Arabidopsis decreases the association of ARFs to polysome. What is the knockdown efficiency for TOR RNAi shown in Figure 3?

Extracts from WT and *TOR RNAi* plants were assayed by western blot and results are now presented as Fig. 4C. The knockdown efficiency for TOR in TOR RNAi seedlings is highly reproducible and is about 80-90%.

The authors need to show that a control RNAi seedling shows similar results as the ones presented in Figure 2."

sqRT-PCR analyses of *ARF/bZIP11* mRNAs in WT/*TOR RNAi* seedlings was done in parallel and we set the individual mRNA level in WT/NAA plants to 100%.

"3- The knockdown of S6K1 or the rescue with expression of a constitutively active mutant of S6K1 in TOR-depleted seedlings (Figure 3) would add strength to the role of TOR/S6K1 on the polysomal loading of uORF-mRNAs."

We currently see no way to conduct ectopic expression of an active mutant of S6K1 in siRNA resistant TOR construct. However, it was possible transiently express high levels of S6K1 (or eIF3h) mutants in Torin-1-treated suspension protoplasts. In this case, transiently produced S6K1 complemented loss of reinitiation. We now present the results of this experiment as Fig. 7D.

The knockdown of S6K1/ S6K2 is under the way in two plant TOR labs (Robaglia and Meyer). The knockout of both is lethal.

"4- Figure 4 and 5 suggest that eIF3h functions downstream of TOR and S6K1 in promoting reinitiation of uORF-mRNAs. Another initiation factor, eIF3b, has been shown to be a target of S6K1 involved in cap-dependent translation in mammals. Is the knockdown of eIF3b able to impair NAA-induced uORF-mRNAs reinitiation?"

eIF3b is a core subunit of eIF3, and its knockdown could strongly affect eIF3 integrity and thus initiation *per se*. Also, the functional role of this phosphorylation would require further investigation.

"5- The authors need to further characterize eIF3h as a potential target of S6K1. Co-immunoprecipitation experiments of S6K1-eIF3h and TOR-eIF3h are missing, and it would be interesting to assess the binding dynamics in response to NAA treatment and in Torin1-pretreated seedlings.

Coimmunoprecipitation experiments were done and now presented as Fig. 6H. Yeast two-hybrid interactions between eIF3h and S6K1, NTOR, CTOR or Raptor are presented in Fig. S6.

Detailed analysis of eIF3h and S6K1 binding dynamics, dissection of eIF3h and S6K1 binding sites, as well as effects of eIF3h phosphorylation on binding kinetics we plan to perform and publish somewhere else.

In vitro kinase assay showing that S6K1 can phosphorylate the putative RXRXXS site under conditions of NAA will be suitable.

Currently active and specific commercial recombinant *At*S6K1 is not available. We are looking for commercial recombinant mS6K1 specific for plants. However, phosphorylation of eIF3h is highly responsive to NAA treatment of WT seedlings (see our 2D gel results in Fig. 6A).

As for S6K1, does the expression of eIF3h S178D in TOR-depleted or Torin1-treated seedlings rescue the decrease in polysomes loading?"

We currently see no way to overexpress eIF3h mutants in siRNA resistant TOR construct. However, it was possible transiently express high levels of eIF3h or its mutants in Torin-1-treated protoplasts. The results are presented as a new Fig. 7D. This reviewer can see that transiently produced eIF3h-S178D rescued expression of *ARF3*-reporter.

6- What is the effect of eIF3h S178A and S178D on root gravitropism?

We do not have full-length eIF3h knockout line, and it is a quite hard job to ectopically overexpress eIF3h-S178D in already severely affected *eif3h-1* plants.

2nd Editorial Decision 01 February 2013

Thank you for submitting a new version of your manuscript to The EMBO Journal. It has now been seen by two of the original referees whose comments are shown below.

As you will see from the reports both referees - including the original ref #1 - express great interest in your findings and support publication of the manuscript pending minor revisions. However, while most of the remaining criticisms can be addressed by clarifying and re-phrasing statements made in the text, referee #1 does ask you to include data for polysome recruitment for the eIF3h phosphomutants and the potential implications for translation re-initation.

Given the referees' positive recommendations, we invite you to submit a revised version of the manuscript, addressing the comments of both reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance or rejection of your manuscript will therefore depend on the completeness of your responses to the full satisfaction of the referees in this revised version. Please do not hesitate to contact me if you have questions related to the review process and the requests made by the referees.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer-Review Process File, and 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.

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

REFEREE REPORTS:

Referee #1

This report concerns target of rapamycin and ribosomal protein S6 kinase in plants, as area which is much less explored than the roles of these protein kinases in mammalian cells. The authors have provided responses to all the points I raised on the original manuscript.

The paper presents novel and interesting data. For example, the data indicate:

(i) That auxin stimulates TOR signaling and the downstream phosphorylation of S6K in Arabidopsis cells in culture. Furthermore, changes in TOR signaling modulate the interactions of the initiation factor eIF3 with S6K and TOR.
(ii) That an auxin analog promotes the association of mRNAs with a uORF on polysomes, without a similar effect on 'control' mRNAs sch as actin; interestingly, this effect is more than reversed by the TOR inhibitor Torin 1.

The authors employ the level of phosphorylation of a specific residue in TOR as a 'read-out' of S6K activity. One concern is that, while a serine residue is present at this position in Arabidopsis TOR, it is not in the widely-accepted consensus for phosphorylation by S6K (RxRxxS/T). I guess it is possible that the substrate specificity of Arabidopsis differs from that of mammalian S6K. At all events, the authors should comment on this in the text.

(iii) Treatment with the auxin analog causes a change in the association of S6K and TOR with the polysome region of the gradient. The authors state that p-TOR is seen at the top of the gradient in Fig. 3A and p-S6K is on polysomes (although S6K isn't) in Fig. 3B. In fact the signals on the westerns are so weak that it is very hard to be sure about this. The authors need to modify their statements about this (which do not seem to be central to their overall conclusions).

It remains confusing that TOR cannot be detected in control or Torin-1 treated sample, only in the NAA set. The authors' earlier work suggested Torin-1 does not affect total TOR levels, but that conclusion is not consistent with the data in Fig. 4D. Why the difference?

(iv) Auxin promotes the phosphorylation of a subunit of eIF3 (eIF3-h) in a manner that is sensitive to inhibition of TOR (Torin-1) and phosphorylated eIF3-h (presumably as part of the eIF3 complex) associates with polysomes. Its polysomal association appears to be controlled through TOR. eIF3-h contains a possible phosphorylation site for S6K and indeed the two can be co-immunoprecipitated.

In Fig. 7D, one assumes the authors are using P-specific antibodies to detect also the 'phosphomimetic' S178D or T449E mutants. It is interesting that the P-specific antibodies seem to cross-react with these mutants, but it is not possible to draw conclusions about relative intensities and the sentence (middle of p. 16) 'In Torin-1 conditions ...' must be removed and heavily altered. One assumes the third lane in each set is for the mutant, but this is not really clear from the way the figure has been made.

(v) The reinitiation of translation after a specific upstream open reading-frame (uORF) is positively regulated by TOR signalling. The data for the eIF3-h S178D and S178A mutants are interpreted to indicate that phosphorylation of eIF3-h at this TOR-regulated site aids reinitiation.

The authors suggest that phosphorylation of eIF3-h may promote association with polysomes and that this may play a role in the reinitiation process. The authors could test this using their S178D and S178A mutants, and examining their association with polysomal material. However, I was unable to find such data. It would significantly strengthen the authors' arguments if they tested this.

(vi) TOR also appears to play a role in gravitropic responses, in particular the distribution of auxin, which is certainly of interest - although the mechanisms involved are unclear.

The Abstract contains a number of abbreviations, some of which are not defined (e.g., S6K1, eIF3h).

Referee #2:

The authors have addressed my comments. Some minor comments below:

Fig 3 with RNAse, TOR and S6K present still in low density fractions after RNAse treatment suggesting association with non ribosome-related complex. Can the authors clarify this particularly since the authors claim Torin1 does not completely inactivate TOR (yet TOR phosphorylation in the RNAase treated samples seem to disappear).

Correction; page 10 title":...TOR in planta maintains inactive (not inactivate)"

Fig 6H: label IP with mock vs IP

Fig 8A: The X axis labels (10, -) are mislabeled

1st Revision - authors' response 12 February 2013

Reviewer 1

The authors employ the level of phosphorylation of a specific residue in TOR as a 'read-out' of S6K activity. One concern is that, while a serine residue is present at this position in Arabidopsis TOR, it is not in the widely-accepted consensus for phosphorylation by S6K (RxRxxS/T). I guess it is possible that the substrate specificity of Arabidopsis differs from that of mammalian S6K. At all events, the authors should comment on this in the text.

Although the TGRDFS^{2448} motif differs from the consensus for phosphorylation by S6K1/ Akt (R/KxR/KxxS/T), the first position seems to be less critical—CDK2-T160 contains the PVRTYT motif, and NMDAR1-S896 is phosphorylated by Akt very efficiently despite the presence of F at the first position (see Cell signalling manual). This is now included in the text (page 9, the first para).

(iii) Treatment with the auxin analog causes a change in the association of S6K and TOR with the polysome region of the gradient. The authors state that p-TOR is seen at the top of the gradient in Fig. 3A and p-S6K is on polysomes (although S6K isn't) in Fig. 3B. In fact the signals on the westerns are so weak that it is very hard to be sure about this. The authors need to modify their statements about this (which do not seem to be central to their overall conclusions).

Our statements are now modified (page 10, first para).

It remains confusing that TOR cannot be detected in control or Torin-1 treated sample, only in the NAA set. The authors' earlier work suggested Torin-1 does not affect total TOR levels, but that conclusion is not consistent with the data in Fig. 4D. Why the difference?

Torin1 does not affect total levels of TOR in plant extracts, but it affects TOR phosphorylation status and thus its binding to polysomes. In Fig. 4D we analyze only polysomes and ribosomal subunits that seem to associate with either inactive S6K1 (TOR RNAi/ Torin-1) or phosphorylated TOR (NAA).

In Holtz et al (2005) the situation is similar: under inactivation conditions S6K1 is bound to eIF3-PIC (TOR is not there), but TOR-P binds eIF3-PIC under activation conditions. According their data, the S6K1 phospho-mimic is not able to interact with eIF3.

Interestingly, reinitiation events are not efficient in order to restrict levels of potent factors that are toxic in high concentrations. Our hypothesis is that TOR up-regulation would trigger their overproduction. It may be not surprisingly that in mock plants inactive S6K1 preferentially binds polysomes (Fig. 3A). The level of phosphorylated TOR is not high and is apparently below or at the limit of detection of our antibodies. In auxin conditions, TOR is up-regulated and thus is seen in polysomes.

In Fig. 7D, one assumes the authors are using P-specific antibodies to detect also the

'phosphomimetic' S178D or T449E mutants. It is interesting that the P-specific antibodies seem to cross-react with these mutants, but it is not possible to draw conclusions about relative intensities and the sentence (middle of p. 16) 'In Torin-1 conditions ...' must be removed and heavily altered. One assumes the third lane in each set is for the mutant, but this is not really clear from the way the figure has been made.

The sentence has been modified. Figures 7C and D have been corrected.

(v) The reinitiation of translation after a specific upstream open reading-frame (uORF) is positively regulated by TOR signalling. The data for the eIF3-h S178D and S178A mutants are interpreted to indicate that phosphorylation of eIF3-h at this TOR-regulated site aids reinitiation.

The authors suggest that phosphorylation of eIF3-h may promote association with polysomes and that this may play a role in the reinitiation process. The authors could test this using their S178D and S178A mutants, and examining their association with polysomal material. However, I was unable to find such data. It would significantly strengthen the authors' arguments if they tested this.

It is quite a hard job to ectopically overexpress eIF3h-S178D or S178A in already severely affected *eif3h-1* plants. Thus, we transformed protoplasts from *eIF3h-1* mutant plants with a plasmid encoding either eIF3h or eIF3h-S178D or eIF3h-S178A followed by polysome isolation. The results are presented in Fig. S7.

The Abstract contains a number of abbreviations, some of which are not defined (e.g., S6K1, eIF3h).

All abbreviations are now defined.

Reviewer 2

Fig 3 with RNAse, TOR and S6K present still in low density fractions after RNAse treatment suggesting association with non ribosome-related complex. Can the authors clarify this particularly since the authors claim Torin1 does not completely inactivate TOR (yet TOR phosphorylation in the RNAase treated samples seem to disappear).

After RNase-treatment, polysomes are divided into 80S and subunits. Actually, the effect of RNase treatment could be more drastic than simple polysomal mRNA degradation, and may affect phosphorylation status. In my opinion, low density fractions represent 40S-containing complexes (80S/60S/40S are somewhat more widely distributed along the gradient), or may include complexes related to other functions of TORC1.

Correction; page 10 title":...TOR in planta maintains inactive (not inactivate)"

The mistake was corrected.

Fig 6H: label IP with mock vs IP

The mistake was corrected.

Fig 8A: The X axis labels (10, -) are mislabelled

The mistake was corrected.