mTORC2 can associate with ribosomes to promote cotranslational phosphorylation and stability of nascent Akt polypeptide

Won Jun Oh, Chang-chih Wu, Sung Jin Kim, Valeria Facchinetti, Louis-André Julien, Monica Finlan, Philippe P. Roux, Bing Su and Estela Jacinto

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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 28 June 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by two referees whose comments to the authors are shown below. The third referee was not able to return his/her report due to unexpected personal circumstances and offers his/her sincere apologies. As you will see both referees consider the study as interesting and would support its publication here after appropriate revision. We should thus be able to consider a revised manuscript if you can address the referees’ criticisms in an adequate manner and to their satisfaction. However, it will be indispensable to provide stronger evidence for your conclusion that the assembled mTORC2 complex interacts with actively translating ribosomes as detailed by referee 2.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript as well as on the final assessment 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 initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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

Editor
The EMBO Journal

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REFEREE COMMENTS

Referee #1 (Remarks to the Author):

In this paper the authors present data on the regulation of protein translation by the TORC2 complex. They demonstrate that TORC2 associates active ribosomes and promotes cotranslational phosphorylation of Akt. This is responsible for induction of Akt phosphorylation at T450, and this is required to prevent immediate ubiquitination and degradation of newly synthesized Akt protein. In summary the authors propose a new role for TORC2 in the association with translating ribosomes and regulation of Akt synthesis and phosphorylation.

Overall this is a sound study from a group who has been actively investigating Akt regulation by TORC signaling. In my opinion the conclusions stated are generally consistent with the data presented, and certainly there is sufficient novelty here for an EMBO J paper. The scope is also appropriate. It is also worth mentioning that the paper is very well presented. I have two questions for the authors to address before this paper can be further considered for publication.

1. Fig. 6. The data do not really show that it is the T450 site whose phosphorylation regulates ubiquitination. I understand this has been shown before, but here an experiment with WT and T450A or T45D Akt might provide a bit more mechanistic insight?

2. At the very end of the study the authors do show some PKC data, but these really only deal with the ubiquitination aspect. I think it would be worthwhile to have at least one or two experiments at the beginning to determine whether PKCs, and not just Akt, are also cotranslationally regulated by TORC2 in the same manner, or if there are any differences? After all this group has indeed published recent papers in EMBO J comparing Akt and PKC regulation by the TORC2 complex.

Referee #2 (Remarks to the Author):

This study examines the mechanism by which the turn motif (TM) on Akt becomes constitutively phosphorylated, which has been shown previously to be dependent on mTORC2. This phosphorylation has been hypothesized to occur co-translationally in Akt and other AGC kinases. HA-mTOR was added during an in vitro translation assay and shown to increase phosphorylation of the TM but not the hydrophobic motif (HM). Monosome/polysome fractionation demonstrates that TM phosphorylation mirrors total Akt levels found in these fractions. Using longer or shorter Akt constructs, it is suggested that the HM is not phosphorylated because it is inaccessible during translation. Like Akt, mTORC2 components can be coIPed with mTor, and this is disrupted upon loss of Sin1 or Rictor. Evidence is provided that mTORC2 associates with rpL23a. Sin1 KO MEFs are found to have defects in protein translation, with reduced polysomes. As published previously, Akt is more ubiquitinated in Sin1-/- MEFs, and this appears to be enriched in the monosome fraction.

This study lends support to an old assumption that Akt and other AGC kinases are phosphorylated on their turn motifs co-translationally or during folding. The in vitro data are relatively convincing, but the data that mTORC2 (as a complex) binds to the actively translating ribosome, as claimed, is preliminary and the model presented is difficult to accept at face value. The stoichiometry of mTORC2 to total ribosomes in the cell is likely to be many orders of magnitude lower, and this difference is exacerbated when one considers the data in this paper (Figure 4A), which demonstrates that, like Akt, less than 1% of total mTOR is associated with ribosome fractions. Therefore,
mTORC2 must be associating with an extremely small fraction of total ribosomes, and one is left wondering how mTORC2 specifically associates with ribosomes that are translating mRNAs encoding its substrates, which themselves are low abundant proteins. Further, due to their high positive charge, ribosomal proteins are inherently sticky, making control and reciprocal IPs with endogenous proteins critical.

Specific Comments:

1. Figure 1B: This assay should be repeated with RICTOR versus RAPTOR IPs to demonstrate the specificity for the two mTOR complexes. Also, how specific is this phosphorylation for T450. This should be tested in a hot assay comparing phosphate incorporation into a newly translated wild-type versus T450A mutant of Akt.

2. Figure 2A: The phosphorylation of these extended constructs in vivo should be assessed. Is the HM constitutively phosphorylated (i.e., independent of growth factors)? T308 should be used as a control.

3. The experiment presented in 2B is uninformative, as the truncated Akt construct is clearly not being fully translated.

4. Figure 4: Given that all newly translated proteins are associated with ribosomal fractions, as demonstrated with Akt in figure 1, it is difficult to conclude that assembled mTORC2 is associated with ribosomes. The conclusion that raptor is not in the polysome fraction is subjective and dependent on the blot exposure. The results in Figures 4B and C are critical because they provide the best evidence that the components of mTORC2 are associating with the ribosomal proteins in a complex-dependent manner. However, given the findings in Figures 5 and 6 that disrupting mTORC2 causes defects in protein translation/"polysome integrity" calls into question the interpretation of the data from Sin1 KO and Rictor knockdown cells. The loss of mTOR binding to ribosomal subunits in these setting could be secondary to disruption of polysomes, as seen in Figure 4A with RNAse treatment for example. In general, more experiments are needed to clearly demonstrate that intact mTORC2 is associating with intact ribosomes.

5. Figure 4D: The effects of these washes on the binding of endogenous Rictor and Sin1 to HA-mTOR need to be shown. It has been shown previously that Triton X-100 will disrupt mTORC2. Therefore, these data conflict with the data in Figure S7B, suggesting that mTORC2 interacts with L23a through Rictor.

6. Can the effects on translation in the Sin1-/- MEFs be rescued by re-expressing Sin1.

7. Figure 6: The differential effects of rapamycin and Torin (and other mTOR kinase inhibitors) on translation and polysome profiles has been shown in several previous studies to be mediated by rapamycin-resistant effects of mTORC1.

8. Figure S7C is missing.

Other Comments:

1. There are a frustrating number of fundamental controls missing from the data shown, which make interpretation difficult.

Examples:

Figure 4C: Obviously a control siRNA is needed to conclude that raptor knockdown is having no effect on this coIP.

Figure 4E: Controls antibodies for these IPs are needed (e.g., raptor), or the same IPs from the relevant knockout lines, and these should be run on the same blot. mTOR levels in these IPs should also be shown.

Supplemental figure 5 needs to be repeated with control IPs shown on the same blot as mTOR IPs.
for all of the proteins shown.

Figure 6C: One needs to know how much Akt and PKCa are in the IP, not the lysate.

Figure 3A is impossible to interpret with regard to HM phosphorylation, since the conclusion made is based solely on the blot exposure shown.

Figure 3C: Quantification of phospho to total Akt for this experiment is needed.

2. As presented, the fractionation traces would not readable if decreased to journal size.

Referee 1:

We are very grateful to this reviewer for his/her highly positive assessment of our studies and encouraging comments. The suggested two experiments indeed address relevant issues to further support our conclusion. The results are now added as Figures 2G and 7B.

In this paper the authors present data on the regulation of protein translation by the TORC2 complex. They demonstrate that TORC2 associates active ribosomes and promotes cotranslational phosphorylation of Akt. This is responsible for induction of Akt phosphorylation at T450, and this is required to prevent immediate ubiquitination and degradation of newly synthesized Akt protein. In summary the authors propose a new role for TORC2 in the association with translating ribosomes and regulation of Akt synthesis and phosphorylation.

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1. Fig. 6. The data do not really show that it is the T450 site whose phosphorylation regulates ubiquitination. I understand this has been shown before, but here an experiment with WT and T450A or T45D Akt might provide a bit more mechanistic insight?

We have now performed this suggested experiment and confirmed that whereas overexpressed wild type Akt does not undergo ubiquitination in the mono/polysome fractions, T450A Akt mutant was significantly ubiquitinated in these fractions (new Figure 7B). These results further support that absence of T450 phosphorylation leads to premature ubiquitination of newly synthesized Akt.

2. At the very end of the study the authors do show some PKC data, but these really only deal with the ubiquitination aspect. I think it would be worthwhile to have at least one or two experiments at the beginning to determine whether PKCs, and not just Akt, are also cotranslationally regulated by TORC2 in the same manner, or if there are any differences? After all this group has indeed published recent papers in EMBO J comparing Akt and PKC regulation by the TORC2 complex.

We have now performed in vitro translation (bacterial components)/kinase assay using PKC-alpha as the substrate. These results are included in Figure 2G and demonstrate that PKC-alpha can be phosphorylated at both the TM and HM sites in vitro during translation only in the presence of HA-mTOR immunoprecipitates.
We would like to stress that previous studies using recombinant PKC as the substrate and HA-mTOR immunoprecipitates failed to detect mTOR-dependent phosphorylation (Ikenoue et al; Facchinetti et al both EMBO J 2008). Our results now demonstrate for the first time that this phosphorylation can be reconstituted efficiently in vitro during PKC-alpha translation.

Referee #2:

We sincerely thank this reviewer for several helpful suggestions and comments to improve the paper. We specifically address the major criticism raised by this reviewer on providing additional evidence that intact mTORC2 associates with translating ribosomes. We have added more experiments to address other specific questions and provided additional control experiments as suggested. We also clarify some points that were confusing or not strongly emphasized in the first submission.

This study examines the mechanism by which the turn motif (TM) on Akt becomes constitutively phosphorylated, which has been shown previously to be dependent on mTORC2. This phosphorylation has been hypothesized to occur co-translationally in Akt and other AGC kinases. HA-mTOR was added during an in vitro translation assay and shown to increase phosphorylation of the TM but not the hydrophobic motif (HM). Monosome/polysome fractionation demonstrates that TM phosphorylation mirrors total Akt levels found in these fractions. Using longer or shorter Akt constructs, it is suggested that the HM is not phosphorylated because it is inaccessible during translation. Like Akt, mTORC2 components can be detected associated with ribosomal fractions. Ribosomal proteins can be coIPed with mTor, and this is disrupted upon loss of Sin1 or Rictor. Evidence is provided that mTORC2 associates with rpl23a. Sin1 KO MEFs are found to have defects in protein translation, with reduced polysomes. As published previously, Akt is more ubiquitinated in Sin1-/- MEFs, and this appears to be enriched in the monosome fraction.

This study lends support to an old assumption that Akt and other AGC kinases are phosphorylated on their turn motifs co-translationally or during folding. The in vitro data are relatively convincing, but the data that mTORC2 (as a complex) binds to the actively translating ribosome, as claimed, is preliminary and the model presented is difficult to accept at face value. The stoichiometry of mTORC2 to total ribosomes in the cell is likely to be many orders of magnitude lower, and this difference is exacerbated when one considers the data in this paper (Figure 4A), which demonstrates that, like Akt, less than 1% of total mTOR is associated with ribosome fractions. Therefore, mTORC2 must be associating with an extremely small fraction of total ribosomes, and one is left wondering how mTORC2 specifically associates with ribosomes that are translating mRNAs encoding its substrates, which themselves are low abundant proteins.

We absolutely agree with this comment from the reviewer that the stoichiometry of mTORC2 to total ribosomes in the cell is much lower. Nevertheless, the fraction that associates with the translating ribosomes cannot be ignored and the finding that mTORC2 is regulating the cotranslational phosphorylation of nascent Akt suggests that it associates with a specific pool of translating ribosomes. We would like to clarify that we do not conclude in the paper that mTORC2 controls or associates with all translating ribosomes.

In the previous submission, we have shown that under our mild lysis condition (Dounce homogenization), there was defective polysome recovery in the SIN1-/- MEFs. We have performed further experiments to address this puzzling observation. If we subject the remaining pellet that is recovered after mild lysis to subsequent Dounce homogenization and addition of CHAPS detergent, we release polysomes in both wild type and SIN1-deficient cells. These new findings reveal that there are indeed abundant polysomes in SIN1-deficient cells (but less compared to wild type) (see Figure 6B).
These results support the notion that a specific pool of translating ribosomes (that is poorly recovered under mild lysis conditions) can associate or are dependent on mTORC2. The identity of this pool of polysomes that mTORC2 associates with, remains to be further elucidated in future studies. However, since the point that we would like to emphasize in Figure 6 is the association of mTORC2 with polysomes, we removed the earlier discussion and data about poor polysome recovery and now only show the efficient polysome recovery under the presence of CHAPs to avoid confusion. The bottomline is that there are recoverable polysomes, although somewhat decreased, in mTORC2-disrupted cells (consistent with previous observations using mTOR inhibitors). Please note that to obtain comparable amounts of polysomes from wild type versus SIN1−/− fractions, we loaded more extracts from SIN1−/− cells. Despite more protein loaded from these mTORC2-disrupted extracts, we obtained significantly less mTOR and rictor colocalizing with polysomes. Interestingly, the mTOR and rictor that can be detected in the monosome fractions (4−6) appear to be modified differently (SDS-PAGE migration is altered), suggesting they are distinct from the assembled mTORC2. These findings are incorporated into the Results and Discussion sections.

“How mTORC2 can specifically associate with ribosomes that are translating mRNAs encoding its substrates” is indeed an important and very interesting question but is beyond the scope of the current studies.

Further, due to their high positive charge, ribosomal proteins are inherently sticky, making control and reciprocal IPs with endogenous proteins critical.

We have added several controls to further demonstrate that interactions of mTORC2 with the ribosomal proteins are specific. As shown in Figure 5C, the association of HA-mTOR with large ribosomal proteins correlates with an intact mTORC2. Figure 5D and 5E also demonstrates that whereas mock IP does not co-precipitate ribosomal proteins, rpL23a and rpL26 specifically co-IP with endogenous rictor and SIN1 but not raptor even in the presence of RNase. In vitro binding assays in Supplementary Figure S7B and S7C also show specific enhanced binding of rpL23 and rpL23a with mTORC2 components from wild type versus SIN1−/− cells. Furthermore, there is little to no binding of rpL23 and very little binding of rpS6 with endogenous mTOR or rictor from SIN1−/− cells despite normal levels of these ribosomal proteins (Figure 5A). Taken together, these results argue against a non-specific interaction of ribosomal proteins with the mTORC2 components. Hence, we do not think that the interaction we observe between mTORC2 components and ribosomal proteins are just due to inherent stickiness of the ribosomes.

Specific Comments:

1. Figure 1B: This assay should be repeated with RICTOR versus RAPTOR IPs to demonstrate the specificity for the two mTOR complexes. Also, how specific is this phosphorylation for T450. This should be tested in a hot assay comparing phosphate incorporation into a newly translated wild-type versus T450A mutant of Akt.

Using our current lysis conditions, we have found that immunoprecipitation of mTOR, rather than rictor, can efficiently phosphorylate Akt during translation. The exact reason for this is unclear at the moment although we suspect that the fraction of the relevant intact mTORC2 complex that could function in cotranslational phosphorylation is higher upon mTOR IP from HEK293 cells. Alternatively, IP of rictor may mask a crucial site that is required for either binding or activity of mTORC2 towards its substrate. Nevertheless, in order to address the specificity for phosphorylation by mTORC2 versus mTORC1, we incubated cellular extracts with either rapamycin (blocks mTORC1) or Torin1 (blocks mTORC1 and mTORC2) before immunoprecipitation of HA-mTOR. By this
method, we found that whereas Akt is still phosphorylated at the TM and HM sites upon rapamycin treatment, we detected no phosphorylation of these sites upon Torin1 treatment (Figure 2F).

We also addressed the specificity of phosphorylation at T450 by mTOR. We mutated this site to alanine and found that phosphorylation of this site but not the HM site is abolished during in vitro translation/kinase assay (Figure 2E).

2. Figure 2A: The phosphorylation of these extended constructs in vivo should be assessed. Is the HM constitutively phosphorylated (i.e., independent of growth factors)? T308 should be used as a control.

We have now examined the phosphorylation of extended constructs in vivo. We created stable cell lines and were able to successfully express HA-Akt and Akt-His. To express Akt-HA-His2X, we performed transient expression. However, not surprisingly, HM phosphorylation can still be induced upon restimulation of starved cells. We believe this is due to the sensitivity of the HM site for dephosphorylation upon withdrawal of growth signals. Thus, under these conditions, we will not be able to technically determine whether the long-tailed Akt underwent cotranslational phosphorylation in vivo. To address cotranslational phosphorylation, we performed polysome purification of cells overexpressing Akt-HA-His2X. Under these conditions, we found HM phosphorylation of long tail but not endogenous Akt colocalizing with translating ribosomes. There was no HM phosphorylation detected in the polysome fractions upon disruption of the ribosomes with RNase. Activation loop phosphorylation did not colocalize with the polysomes. This new figure is now added as Figure 3D.

3. The experiment presented in 2B is uninformative, as the truncated Akt construct is clearly not being fully translated.

We have optimized this assay by creating a new truncated Akt construct that is more efficiently translated than the previous construct that we used. The results in new Figure 2C support our conclusion that inaccessibility of the phosphorylation site (due to TM truncation) during translation prevents phosphorylation.

4. Figure 4: Given that all newly translated proteins are associated with ribosomal fractions, as demonstrated with Akt in figure 1, it is difficult to conclude that assembled mTORC2 is associated with ribosomes.

This comment from the reviewer is indeed a point that we have considered carefully. The strongest evidence that demonstrates the presence of assembled mTORC2 and not nascent mTORC2 component polypeptides in the polysomes is shown in Figure 6A wherein we detected phosphorylation of mTOR at Ser2481 in the polysome fractions. Phosphorylation at this site is a marker for assembled mTORC2 and presence of mTOR complex activity (Copp et al Cancer Res., 2009; Soliman et al, JBC, 2010). Hence, the phosphorylated mTOR in the polysome fractions represents intact mTORC2 and not newly synthesized mTOR. Another strong evidence to support association of assembled mTORC2 with ribosomes is now presented in Figure 6C wherein we analyzed association of mTOR and rictor with the polysomes in wild type versus SIN1-/- cells. Indeed, we confirmed that there is dramatically decreased levels of mTOR and rictor in polysomes from SIN1-/- MEFs (even upon adjusting amounts of ribosome proteins in the polysome fractions) (see also discussion above on page 3).

The conclusion that raptor is not in the polysome fraction is subjective and dependent on the blot exposure.

The exposure of each of the blots shown in this figure corresponds to the
longest exposed blot. Also, we based our conclusion on the relative amounts of the bands from each group of fractions. For example, despite abundant mTOR in the C fractions of both (-) and (+) RNase groups, there is much more mTOR found in the M fraction from (-) rather than (+) RNase group. This is even more obvious in rictor wherein high amounts were found in the M and P fractions of (-) RNase group. Thus, even if we overexpose both (-) and (+) RNase blots, there will also be greater amounts of rictor in M and P fractions in (-) RNase group. In contrast, although raptor is detected slightly in the polysome fractions, unlike mTOR, rictor, and SIN1, the pattern of raptor expression is very similar in non-RNase versus RNase treated extracts. In contrast, SIN1, mTOR and particularly rictor are found in more abundant amounts in the high density polysome fractions and clearly colocalized to low density fractions upon RNase treatment. Hence, we conclude that the raptor in the high-density fractions is not polysome-associated. We have now quantitated the levels in each of the fractions to more clearly demonstrate the difference in amounts of mTORC components in the fractions (see lower panels Figure 6A). This, together with additional evidence provided in this revised version that raptor does not associate with rpL23a and rpL26 support our conclusion that mTORC2, but not mTORC1 associates with ribosomes (Figure 5E). Together, these results clearly demonstrate that mTORC2 components associate with translating ribosomes.

The results in Figures 4B and C are critical because they provide the best evidence that the components of mTORC2 are associating with the ribosomal proteins in a complex-dependent manner. However, given the findings in Figures 5 and 6 that disrupting mTORC2 causes defects in protein translation/"polysome integrity" calls into question the interpretation of the data from Sin1 KO and Rictor knockdown cells. The loss of mTOR binding to ribosomal subunits in these setting could be secondary to disruption of polysomes, as seen in Figure 4A with RNase treatment for example. In general, more experiments are needed to clearly demonstrate that intact mTORC2 is associating with intact ribosomes.

We thank the reviewer for raising this important point. Firstly, we revised the text to more accurately state the conclusion of the experiment in old Figure 4B and 4C (New Figure 5A and 5B). The conclusion from these experiments is that an intact mTORC2 is required for association of mTOR and rictor with ribosomal proteins. Whether this interaction is due to an assembled polysome cannot be concluded from these experiments but is now addressed in a new experiment that we added and discussed above (see comment #4; New Figure 6C).

5. Figure 4D: The effects of these washes on the binding of endogenous Rictor and Sin1 to HA-mTOR need to be shown.

Done as suggested.

It has been shown previously that Triton X-100 will disrupt mTORC2. Therefore, these data conflict with the data in Figure S7B, suggesting that mTORC2 interacts with L23a through Rictor.

We only used 0.1%TX-100 in these experiments (Figure 4D). Previous report on mTORC2 complex disruption by TX-100 used 1%. We extended this figure to confirm that using 1%TX-100 disrupts mTORC2 and weakens association of mTOR with rpL23a. These results do not disagree with old Figure S7B (New Figure S6C) that an intact mTORC2 is required for stable and enhanced interaction with rpL23.

6. Can the effects on translation in the Sin1-/- MEFs be rescued by re-expressing Sin1.

Yes. The results are now added on Figure 4A.

7. Figure 6: The differential effects of rapamycin and Torin (and other mTOR kinase
inhibitors) on translation and polysome profiles has been shown in several previous studies to be mediated by rapamycin-resistant effects of mTORC1.

The effect on translation of Torin1 in Thoreen et al (JBC, 2009) was measured only at short time point (30 min). In SIN1-deficient cells, we did not observe a significant difference in translation at 30 min but found diminished translation at longer time points (Figure 4A). We found that the defects in translation at 60 min are further exacerbated by Torin1 in SIN1-deficient cells (Supplemental Figure S5), confirming that both mTORC1 and mTORC2 function in translation. As far as we are aware, the effect on translation that was attributed to the rapamycin-resistant effects of mTORC1 was largely based on the phosphorylation of the mTORC1 target 4E-BP (Thoreen et al). As for the effect on polysomes, Yu et al (Cancer Res., 2009) also observed decreased polysomes using WAY-600. Dowling et al (Science, 2010) also observed increased 40S and 60S and decreased polysomes in Torin1-treated cells as compared to 4E-BP1 DKO MEFs. They concluded that Torin1 impaired translation to a greater extent than in 4E-BP DKO MEFs. In this revised version, we did not include the Torin1 effect on polysome profile, since by using the new lysis procedure (inclusion of CHAPS), we obtained similar profile as described by Yu et al. Hence, collectively, our results on translation and polysome profiles are in agreement with the above studies that have examined mTOR inhibitors. Our results do not disagree that there are rapamycin-independent functions of mTORC1.

8. Figure S7C is missing.

We apologize for this oversight. This figure was inadvertently omitted during initial submission. It is now added as New Figure 5B.

Other Comments:

1. There are a frustrating number of fundamental controls missing from the data shown, which make interpretation difficult.

Examples:

Figure 4C: Obviously a control siRNA is needed to conclude that raptor knockdown is having no effect on this coIP.

We do not see an effect of scrambled siRNA on raptor nor rictor response. Unfortunately, we could not repeat this same exact assay using rpL23 antibody since this antibody was originally obtained from the lab of Dr. Y. Zhang and is currently unavailable. We tried commercial sources but they were quite “dirty” and inconclusive. Hence, we moved this Figure to Supplementary Figure 7A. However, we now show that knockdown of rictor but not raptor nor control siRNA can diminish association of mTOR with another large ribosomal protein rpL23a and rpL26 (new Figure 5B).

Figure 4E: Controls antibodies for these IPs are needed (e.g., raptor), or the same IPs from the relevant knockout lines, and these should be run on the same blot. mTOR levels in these IPs should also be shown.

A control (mock) IP is now included in this experiment (New Figure 5D and New Figure 5E). mTOR levels are also now added. IP of rictor or SIN1 from SIN1-/- cell line is shown in Supplemental Figure S6. Currently, we do not have rictor-/- cell line. The rictor-/- cells that we previously used in Facchinetti et al (EMBO J, 2008) were generously provided as primary cells by Dr. K. Guan. These cells are not available to us at the moment.

Supplemental figure 5 needs to be repeated with control IPs shown on the same blot as mTOR IPs for all of the proteins shown.

Done as suggested.
Figure 6C: One needs to know how much Akt and PKCα are in the IP, not the lysate.

In the initial figure on Akt ubiquitination, we had difficulty detecting immunoprecipitated Akt since it was masked by the IgG. We have repeated this experiment and used a different Akt antibody in order to distinguish immunoprecipitated Akt (Figure 7A). The PKCα band however comes from total immunoprecipitated PKCα as we now have corrected.

Figure 3A is impossible to interpret with regard to HM phosphorylation, since the conclusion made is based solely on the blot exposure shown.

We now include the maximum exposure of the HM blot that we obtained. Upon longer exposure, a band on the Torin-treated lane starts to appear and is only slightly less than the non-Torin-treated lane. Therefore we conclude that the phosphorylated HM in the absence of Torin is very minimal.

Figure 3C: Quantification of phospho to total Akt for this experiment is needed.

Done as suggested (now New Figure 3E). We also quantitated other blots in addition to this figure and show averages and S.D. from at least 3 independent experiments (Figures 4D, 4F).

2. As presented, the fractionation traces would not readable if decreased to journal size.

We have re-traced the profiles and these new traces should be readable now.

2nd Editorial Decision  12 October 2010

Thank you for sending us your revised manuscript. Our original referees have now seen it again, and you will be pleased to learn that in their view you have addressed their criticisms in a satisfactory manner, and that the paper will therefore be publishable in The EMBO Journal.

Still, referee 2 suggests a few minor text changes (see below) and I would suggest addressing these in an amended version of the manuscript text.

In addition, there is one remaining editorial issue that needs further attention. Prior to acceptance of every paper we perform a final check for figures containing lanes of gels that are assembled from cropped lanes. While cropping and pasting may be considered acceptable practices in some cases (please see Rossner and Yamada, JCB 166, 11-15, 2004) there needs to be a proper indication in all cases where such processing has been performed according to our editorial policies. Please note that it is our standard procedure when images appear like they have been pasted together without proper indication (like a white space or a black line between) to ask for the original scans. In the case of the present submission the following panel does not fully meet these requirements: supplementary figure S7C. I therefore like to kindly ask you to send us a new, corrected version of this figure. I think that it would also be important to explain in the figure legend that all lanes come from the same gel. Please be reminded that according to our editorial policies we also need to see the original scans for the panel in question.

I am sorry to have to be insistent on this at this late stage. However, we feel that it is in your as well as in the interest of our readers to present high quality figures in the final version of the paper.

Thank you very much for your cooperation.

Yours sincerely,
Editor
The EMBO Journal

REFeree COMMENTS

Referee #1 (Remarks to the Author):

N/A

Referee #2 (Remarks to the Author):

This revised manuscript is greatly improved and provides more convincing evidence that a population of mTOR, likely within mTORC2, can be found associated with polysome fractions, where it can phosphorylate the turn motif of its kinase substrates. A few textual issues should be addressed.

Despite claims to the contrary, the autophosphorylation of mTOR-S2481 is NOT a specific marker of mTORC2. This has been demonstrated to also occur within mTORC1 and be induced by Rheb (e.g., Acosta-Jaquez MCB 2009). Therefore, it is troubling that the authors' indicate that the presence of S2481 phosphorylation in polysome fractions provides their "strongest evidence" that intact active mTORC2, but not mTORC1, is present at polysomes. The authors should dampen their claims based on this evidence in the text on pages 12 and 13. It could be noted that the finding of mTOR-S2481 phosphorylation at polysomes provides evidence supporting the presence of active mTOR (alone or in some complex), rather than nascent mTOR, in polysome fractions.

The title is misleading and seems to suggest that the primary localization of mTORC2 is at ribosomes, which the data clearly demonstrate is not the case. The title could more accurately reflect the data presented with a subtle change: "mTORC2 CAN associate with ribosomes to....." or better yet "A POPULATION OF mTORC2 associates with...". This is an important distinction that should be made to avoid confusion amongst those not directly in this field.

I have now submitted the amended manuscript online including the high resolution figures and amended Supp fig 7C. I relabeled that figure and separated the input from the pull-down blot since we do not have them run in one gel. I can still provide you with the original scans/blots if you need them.

Please let me know if this is acceptable now or if there are other issues.

Thank you for uploading your amended manuscript. I have now had a chance to look into supplementary figure S7 again. I think that as long as pulldown-efficiency compared to input are not the point you would like to make the new figure S7C is fine as the two input lanes come from the same gel. We will therefore not need to see the primary data. Looking at supplementary figure S7B it appears that here the situation is comparable. Do the input lanes that you separated by the black bar also come from a second (but common) gel? If yes, I would suggest putting the panel together in a similar way as S7C. If all lanes come from the same gel it will be important to mention that in the figure legend. Then the panel will not need to be re-arranged.

Could you look into this and upload a modified version of S7, please? Thank you very much.

Yours sincerely,
Editor  
The EMBO Journal  

Additional correspondence  
13 October 2010  

I uploaded the new amended Supplementary Figures. So 7B and 7C are labeled similarly now. The conclusion of that experiment is that mTORC2 but not raptor can bind the large ribosomal proteins (particularly L23a). We actually have another experiment that combined both B and C and run in 1 gel but the problem was the signals were not as robust (although conclusion is the same) that's why I hesitated to use that one. I'm attaching it here in this mail for your evaluation.

2nd Editorial Decision  
14 October 2010  

Thank you for sending us the final amended version of your manuscript. I think that all issues have now been addressed adequately. Regarding figure S7 I think that we can keep panels B and C separately as you have done it now. No further action will be needed in this respect.

The paper will now be publishable in The EMBO Journal and you will receive a formal acceptance letter shortly.

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,

Editor  
The EMBO Journal