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mTORC2 phosphorylates protein kinase Cζ to regulate its stability and activity

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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.)

Editor: Nonia Pariente

1st Editorial Decision 24 April 2013

Thank you for your submission to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, although all the referees find the topic of interest, they also raise a number of concerns that should be experimentally addressed to strengthen the study before publication in EMBO reports can be considered.

Given that all referees provide constructive suggestions on how to make the work more conclusive, I would like to give you the opportunity to revise your manuscript. From reading the comments and after further discussion with the referees, it is clear that the phenotypes you report should be rescued by the reintroduction of Rictor and verification provided using mTOR knockdowns and reconstitution. Although providing these in all the settings referee 3 requests them is probably not required to make your point, some would need to be performed as controls for your existing data (at a minimum, Rictor rescue experiments in figure 1). It is also clear that strengthening the link to actin cytoskeleton remodeling through Rho GTPases, through the use of both phosphomimetic and non-phosphorylatable mutants and careful quantification and statistics (which should be strengthened throughout the study) is a requirement for publication.

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In addition, referee 3's point 1 should be addressed by analyzing different a range of stimuli (and discussing the insulin data), the phosphorylation of relevant in vivo substrates should be analyzed if feasible, and proof of a direct interaction between PKCzeta and mTOR/Rictor should be attempted. We would strongly encourage you to analyze the effect of mTORC2 on PKCiota phosphorylation, which would increase the significance of the work, given that the mTORC2 had been shown to phosphorylate Akt and other PKCs, detracting from the conceptual novelty of the work. However, addressing this point would not be a precondition for acceptance.

If the referee concerns can be adequately addressed, we would be happy to consider your manuscript for publication. However, please note that it is EMBO reports policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

Revised manuscripts must be submitted within three months of a request for revision unless previously discussed with the editor; they will otherwise be treated as new submissions. Revised manuscript length must be a maximum of 28,500 characters (including spaces). When submitting your revised manuscript, please also include editable TIFF or EPS-formatted figure files, a separate PDF file of any Supplementary information (in its final format) and a letter detailing your responses to the referees.

We also encourage the publication of original source data -particularly for electrophoretic gels and blots- with the aim of making primary data more accessible and transparent to the reader. If you agree, you would need to provide one PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures. The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files and should be uploaded when you submit your final version. If you have any questions regarding this please contact me.

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, do not hesitate to get in touch with me if I can be of any assistance.

Referee #1:

Li and Gao demonstrate that PKCz is phosphorylated by mTORC2 at the conserved turn motif site. They provide both in vitro and in vivo evidence to support their conclusion. Furthermore, they provide some evidence that PKCz could mediate the function of mTORC2 in actin cytoskeleton reorganization.

The studies support the function of mTORC2 in the regulation of the turn motifs of AGC kinases and its role in stabilizing and promoting activity of this family of kinases. They also revealed that another PKC (previous studies have shown that conventional PKC can mediate this mTORC2 function) is also involved in actin cytoskeleton reorganization. The data are quite clear and robust and they provide convincing evidence that the TM of PKCz is phosphorylated by mTORC2.

Other issues that can be addressed:
1. Are there known PKCz relevant in vivo substrates that they can examine to support that PKCz TM phosphorylation is required for activation?
2. The data on lamellipodia analysis should be quantitated (counting the number of cells that exhibit this response when overexpressing the mutant). They should also include the Ala mutant.

Referee #2:

Answers to specific questions in instructions to reviewers:

1. YES. The authors identify TORC2 as the turn motif kinase for PKCzeta.

2. YES. It is important that we confirm the kinases responsible for atypical PKC maturation. Despite TORC2 implication as the kinase for many related family members from yeast to mammals, a role in atypical PKC has not been formally demonstrated
3. YES. The atypical PKC kinases are of critical importance in many physiological and pathophysiological settings and as such understanding the pathways responsible for their activation is of broad significance.
4. NO. While significant evidence is reported implying that TORC2 targets atypical PKC zeta, the data is often thin particularly with respect to quantitation, mechanism and phenotypic analysis.

Report

The authors report identification of the mTOR complex TORC2 as the kinase responsible for atypical PKC phosphorylation on the turn motif (T560). They go on to examine a role for aPKC in regulation of Rho family members and the actin cytoskeleton. While TORC2 has been identified as the turn-motif kinase for a number of AGC family members (including various PKC isoforms) a role in the regulation of aPKC has not been formally demonstrated. This finding is therefore novel and significant. There are however some shortcomings to the manuscript. The main criticism is that the experiments do not appear sufficiently quantified or repeated; quantitation requires multiple repeats and statistical analysis rather than the single quantitations presented throughout. Secondly, the data concerning regulation of the cytoskeleton is very limited and perhaps therefore open to over interpretation. The paper would also be significantly strengthened if the experiments were extended to include PKCiota (whether it turns out to be a TORC2 substrate or not), particularly given the key role of this other family member in development and cancer.

Specific Points:

1. As the authors have access to many tools for examining TORC2, including Rictor deficient cells, a role for TORC2 in the regulation of PKCiota should be included. If the required phospho-specific antibodies are not available, surrogate measures could be used such as protein stability or kinase activity. Such assays are applied to PKC zeta and broadening the findings to cover PKC iota would significantly increase the broad biological significance of the study.
2. TORC2 has long been implicated in the organisation of the actin cytoskeleton (initially from yeast genetics) and understanding the mechanism is of key importance to the field. Consequently the regulation of Rho family members and the actin cytoskeleton by PKC zeta requires further experimentation before the strong conclusions given here can be made. Rho activation must be properly quantified with statistics and additional experiments should be conducted to verify a role for PKC zeta in the regulation of the cytoskeleton downstream of TORC2. Here a single PKC zeta mutant (T560D) transfected cell is provided as evidence (Fig 4C).
3. The relative effects of inhibition of the PI3 kinase pathway and mTOR are examined. However the experiments are insufficient to support the conclusions in the text. PKC priming phosphoryaltions are inherently stable when compared to the acutely regulated phosphorylation of PKB. This is tacitly recognised by the long timecourse of TOR inhibition required to reduce turn phosphorylation (Fig 1E). The timecourse for PI3 kinase inhibition (Fig 1B) is insufficient to exclude this pathway as turn motif occupancy is unlikely to be significantly affected after a single hour of inhibition; longer time points could be presented.
4. Turn motif phosphorylation is thought to allow maturation and stabilisation of PKC and the authors report a role for TORC2 in the stabilisation of PKC zeta through this mechanism. There does not however appear to be any affect on basal PKC zeta expression or indeed A-oop phosphorylation in response to loss of PKC zeta phosphorylation. This is somewhat surprising and should at least be discussed.
5. The key anti-PKCzeta phospho-560 antibody does not appear to be defined in the materials and methods. If this is a newly derived antibody, characterisation might be included in supplementary.

Referee #3:

The manuscript by Xin et al. reports that PKCζ is phosphorylated directly by the mTORC2 complex and shows that this phosphorylation event is required for maintaining the kinase activity and protein stability of PKCζ. The authors also claim that mTORC2 regulates the activity of Rho family of

GTPases by controlling PKCζ activity. The findings are of potential interest but there are numerous concerns about the data and their interpretation.

1. Fig 1A: The authors have to demonstrate that, in a reconstitution experiment, the re-expression of Rictor in Rictor KO MEFs can rescue the PKCζ phosphorylation in T560. The authors report that PKCζ is a new substrate for mTORC2 like AKT. However, in contrast to the case of Akt, insulin does not induce PKCζ phosphorylation (p560) (p473). An explanation of this paradoxical observation is required with support of experiment evidences. The authors stimulate with insulin for 15 min. What happens at later times of incubation (20, 30 min)? Could p560 be regulated by other growth factors or serum?

2. Fig 1C: The authors fail to demonstrate the direct interaction between PKCζ and mTOR/Rictor. Although the immunoprecipitation experiments show the co-IP of PKCζ with the mTORC2 complex, no evidence is provided for a physical interaction between PKCζ and mTOR or Rictor. Moreover, the signal from the line 2 is very weak to be convincing.

3.Fig 1F: It would be necessary to show, in a reconstitution experiment, that re-expression of mTOR or Rictor can rescue the PKCζ phosphorylation in mTOR- or Rictor-downregulated cells.

4.Fig 2B: The authors show that in Rictor KO MEFs there is a reduction in PKCζ activity, however it would be necessary to demonstrate that the re-expression of Rictor in these cells can rescue PKCζ activity. Also, what happens in mTOR-downregulated cells?

5. Fig 2C: In Fig 2A the expression of T410A or T560A did not affect the phosphorylation at T560 and T410, respectively. However in the Figure 2C, the expression of T560A produce a reduction in the PKCζ phosphorylation at T410. How is this explained?

6. Fig 3D: Reconstitution with Rictor in the Rictor KO MEFs is required in order to know if the phosphorylation of PKCζ by Rictor is important in the stability of PKCζ.
Also, quantitation of data from replicate experiments and appropriate statistical analysis is needed.

7. Fig 3F: The authors show that the lack of phosphorylation in the phosphorylation deficient mutant (T560A) produces an increment in the PKCζ ubiquitination, but what happens in the phospho-mimetic mutant?

8. Fig 3G: It would be necessary to demonstrate that the re-expression of Rictor in the Rictor KO cells reduces the ubiquitination PKCζ.Furthermore, what happens in mTOR-downregulated cells?

9.Fig. 4C-4D: In order to demonstrate that PKCζ phosphorylation is important in the organization of actin cytoskeleton, the authors have to compare Rictor KO MEFs reconstituted with the phosphorylation-deficient mutant (T560A) and with the phospho-mimetic mutant (T560D). Note that this figures only show show one or just few cells. It is necessary to see more examples here and quantify the results.

10. Fig 4F: Reconstitution with phosphorylation deficient mutant (T560A) is required in order to know if the phosphorylation in T560 (p560) is necessary to rescue the defect in Rac1 and Cdc42 in Rictor KO MEFs.

1st Revision - authors' response 22 August 2013

Response to Editor's Requests:

1) It is clear that the phenotypes you report should be rescued by the reintroduction of Rictor and verification provided using mTOR knockdowns and reconstitution. Although providing these in all the settings referee 3 requests them is probably not required to make your point, some would need to be performed as controls for your existing data (at a minimum, Rictor rescue experiments in figure 1).

We thank the reviewer for pointing out this critical control. In the revised manuscript, we have included a new experiment in which Rictor null ($Ric^{-/-}$) MEF cells were transiently transfected with a Myc-tagged human rictor expression construct. As shown in the revised Figure 1A, re-expression of Myc-Rictor largely rescued the phosphorylation of PKCz at T560 site. The extent of T560 phosphorylation was lower than that observed in WT MEF cells. This is likely due to the limited transfection efficiency in MEF cells. As a control, insulin-stimulated phosphorylation of Akt at S473 was restored in $Ric^{-/-}$ MEF cells expressing Myc-Rictor.

2) It is also clear that strengthening the link to actin cytoskeleton remodeling through Rho GTPases, through the use of both phosphomimetic and non-phosphorylatable mutants and careful quantification and statistics (which should be strengthened throughout the study) is a requirement for publication.

To address these issues, we have added the following new data in the revised manuscript:

(a) $Ric^{-/-}$ MEF cells were transiently transfected with EGFP-tagged PKCz/T560A mutant, and the actin cytoskeleton organization as well as the expression of EGFP-T560A were analyzed using immunofluorescence microscopy. In the revised Figure 4A, immunofluorescence images of actin and EGFP-T560A reveals that the phosphorylation-deficient PKCz was unable to rescue the actin cytoskeleton remodeling defect observed in $Ric^{-/-}$ MEF cells.

(b) Rac1/Cdc42 and Rho activation assays were performed in WT and $Ric^{-/-}$ MEF cells transfected with vector or PKCz/T560A mutant. In contrast to PKCz/T560D, the expression of PKCz/T560A mutant had no effect on the activation of Rac1, Cdc42, or RhoA in $Ric^{-/-}$ MEF cells (Supplemental Figure S2B), suggesting that the phosphorylation at T560 is required for PKCz function.

(c) The experiments shown in the revised Figure 4C were quantified, and the statistical analysis was performed to compare the extent of Rac1, Cdc42 and Rho activation. The new Figure 4D shows that the activation of Rac1 and Cdc42 was significantly decreased whereas RhoA activation was significantly increased in $Ric^{-/-}$ MEF cells; and overexpression of PKCz/T560D mutant rescued the defect in the activation of small GTPases in $Ric^{-/-}$ MEF cells.

(d) As suggested by Reviewer #1, the percentages of cells with lamellipodia were quantified in WT and $Ric^{-/-}$ MEF cells as well as $Ric^{-/-}$ cells expressing PKCz/T560D and PKCz/T560A mutants. This result is now shown in Supplemental Figure S2A.

3) In addition, referee 3's point 1 should be addressed by analyzing different a range of stimuli (and discussing the insulin data), the phosphorylation of relevant in vivo substrates should be analyzed if feasible, and proof of a direct interaction between PKCzeta and mTOR/Rictor should be attempted.

To this end, we investigated the time course of PKCz phosphorylation at both T410 and T560 sites upon serum or EGF stimulation. Consistent with our results obtained with insulin, both T410 and T560 sites were basally phosphorylated in WT MEF cells and serum or EGF had little effect on further promoting phosphorylation of PKCz (Supplemental Figure S1). This is different from S473 phosphorylation of Akt in which a rapid phosphorylation was induced by both serum and EGF stimulation. More importantly, T560 of PKCz was not phosphorylation in $Ric^{-/-}$ cells. As indicated in our manuscript, our results suggest that T560 phosphorylation is insensitive to PI3K activity but requires the presence of rictor. Consistently, mTORC2-mediated phosphorylation of Akt turn motif (T450), an equivalent site of T560 in PKCz, is constitutive and insensitive to PI3K activity as well. We have added the description and discussion of these new data in the revised manuscript.

Regarding the in vivo substrate of PKCz, we did not find any suitable target that can be used to specifically assess PKCz activity in cells. For example, several reported substrates of PKCz can be phosphorylated by multiple PKC isoforms as well. Given that other PKC isoforms are also regulated by mTORC2, it would be difficult to determine in rictor-deficient cells which PKC isoform is responsible for regulating the potential substrate.

Also, it is technically challenging to determine the direct interaction between mTORC2 complex and PKCz. The mTORC2 complex contains at least 6 known proteins including mTOR and rictor. Expression and purification of mTOR, rictor and other protein components in the complex have not been achieved experimentally. The co-immunoprecipitation experiment shown in our manuscript has been utilized to determine the interaction between mTORC2 and its other substrates. In addition,

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the co-immunoprecipitation experiment allows the assessment of binding specificity, as we showed in Figure 1C that PKCz preferentially binds to mTORC2 rather than mTORC1 complex.

4) We would strongly encourage you to analyze the effect of mTORC2 on PKCiota phosphorylation, which would increase the significance of the work, given that the mTORC2 had been shown to phosphorylate Akt and other PKCs, detracting from the conceptual novelty of the work. However, addressing this point would not be a precondition for acceptance.

In the revised manuscript, we determined if the turn motif of PKCi is phosphorylated by mTORC2. Since we did not detect any PKCi expression (the mouse homologue is also called PKCl) in WT or $Ric^{-/-}$ MEF cells using the PKCi/l specific antibody, we treated 293T cells with mTOR inhibitor PP242. Similar to PKCz, the turn motif phosphorylation of PKCi was completely lost in mTOR inhibitor-treated cells whereas the activation loop was not affected (Supplemental Figure S1C), thus suggesting that the turn motif of PKCi (T564 in human PKCi) is also a substrate of mTORC2. However, due to lack of detectable expression of PKCi in MEF cells, the subsequent data obtained in MEF cells are considered PKCz specific. Taken together, our findings identified mTORC2 as an upstream regulator of atypical PKCs, including PKCz and PKCi, by directly controlling the TM phosphorylation.

In addition, we determined the ubiquitination status of T560D mutant, and the new data are now shown in revised Figure 3F.

2nd Editorial Decision — 11 September 2013

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the enclosed reports from referees 1 and 2, who were asked to assess your revised study. As you will see, although they both consider the study much improved, they have several outstanding minor concerns which should be addressed before we can consider publication of your study. Given that they are easily addressable, we have decided to open an exceptional second round of revision in this case.

As noted by referee 1, the study should incorporate discussion of several points and rewording of a couple of sentences. Referee 2 has a more substantial concern, which was brought up in the initial round of review and needs to be addressed, and relates to a better quantification of the lamellipodia and stress fiber data. In addition s/he has two other minor points.

In going through your manuscript, I have noted that you need to expand the Materials and Methods section. Please note that basic Materials and Methods required for understanding the experiments performed must remain in the main text, although additional detailed information may be included as Supplementary Material.

Lastly, we now encourage the publication of original source data -particularly for electrophoretic gels and blots, but also for graphs- with the aim of making primary data more accessible and transparent to the reader. Please provide one PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures and an Excel sheet or similar with the data behind the graphs. The files should be labeled with the appropriate figure/panel number, and the gels should have molecular weight markers; further annotation could be useful but is not essential. The source files will be published online with the article as supplementary "Source Data" files and should be uploaded when you submit your final version. If you have any questions regarding this please contact me.

I look forward to seeing a new revised version of your manuscript as soon as possible, and always within 4 weeks, as a final decision on your study has to be made before 24th Oct 2013 (6 months from our initial decision letter).

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Referee #1:

In this revised submission, the authors have added more evidence to support that rictor is required for the turn motif (TM) phosphorylation of PKC-zeta. They have added rictor reconstitution experiments to demonstrate that phosphorylation at the TM can be restored in rictor-deficient MEFs. They have also included evidence that the related PKC-iota could also be phosphorylated at the TM by an mTORC2-dependent mechanism. They have also added analysis of the effect of overexpression of Ala mutant of PKC-zeta on actin cytoskeleton reorganization and Asp mutant on PKC-zeta ubiquitination. They provided quantitation of the cytoskeleton imaging results and GTPase activation results.

Some issues remain unresolved such as the lack of putative downstream PKC-zeta target and the nature of constitutive phosphorylation of the TM. These unresolved issues should be included in the discussion at least. They should cite examples of these putative substrates based on what they have examined even if they are overlapping with other PKC isoforms. Substrates that could have relevance to actin cytoskeleton reorganization or GTPase activation would be particularly interesting. The authors should also mention in the discussion that the constitutive nature of phosphorylation of the TM (of Akt and PKC) was previously demonstrated to be due to occurrence of this mTORC2-mediated phosphorylation during translation (see Oh et al, EMBO J 2010), hence could be a similar mechanism for PKC-zeta.

Other minor issues:
1. They should avoid stating that "PKC-iota is a substrate of mTORC2". They did not directly demonstrate this, they only showed that the TM phosphorylation of PKC-iota is sensitive to mTOR inhibitors.
2. Error bars should be added in Fig S2A.

Referee #2:

Thank you for requesting my appraisal of the revised manuscript. In general I feel the authors have responded well to the criticisms of the reviewers and the manuscript in my opinion is improved. Most of the points raised have been addressed adequately.

Specifically in response to your request for an opinion on the comments made by reviewer 3: I feel that some of the criticisms raised are somewhat difficult to address. In particular, I have issues with point 1 and 2. With point 1, it is well established for other PKC family members that TORC2 phosphorylation is often not acutely regulated in contrast to PKB. There is therefore no contradiction in the results. Point 2, discusses interaction of PKCzeta with TORC2; again the literature highlights the difficulties associated with such experiments and I would have been surprised to see a robust demonstration of this interaction. There has none the less been a report about rictor-zeta interaction and this may be of relevance to the impact of this study (see below). I also agree totally with your point in the letter to the authors that responding to all the Rictor reconstitution experiments requested by reviewer was not strictly necessary.

My only remaining concern remains with the cytoskeletal work. I think the lamellipodia quantification data helps and could be usefully included in the figure 4 rather than supplementary. I am still slightly concerned about just showing a few cells as evidence for a major role - as I touched on in my original review. Better quantitation of microscopy is achievable - stress fibre quantitation, transfected vs non-transfected numbers. While this has not caused me to reject this revised version it remains a weak point in the paper in my mind.

Minor note: The authors might try looking at LLGL2 as a potential in vivo substrate for PKCzeta.

I might also bring the following paper to the attention of both you and the authors; I don't believe it is referenced but it is certainly relevant and might affect impact.

Cancer Res. 2010 Nov 15;70(22):9360-70. mTOR complex component Rictor interacts with PKCzeta and regulates cancer cell metastasis.
Zhang F, Zhang X, Li M, Chen P, Zhang B, Guo H, Cao W, Wei X, Cao X, Hao X, Zhang N.

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If you have any further requests, please don't hesitate to contact me.

2nd Revision - authors' response 08 October 2013

Response to Editor's Requests and Reviewers' Comments:

1. Both reviewers have indicated that better quantification of immunofluorescence staining results is needed. Specifically, the editor has requested that "Please include the lamellipodia data, and provide images and analysis of more cells, quantitating stress fibers and transfected vs non-transfected populations."

To address this, we have preformed more detailed quantification of cells with either lamellipodia or stress fibers. Due to the space limitation, we divided the original Figure 4 into two figures and added quantification results in new Figures 4 and 5. These data are now shown in Fig. 4C and Fig. 5E. The numbers of cells quantified and the results of statistical analysis are specified in the corresponding figure legends:

Figure 4. **(C)** The percentages of WT and $Ric^{-/-}$ cells with lamellipodia or stress fibers as indicated by actin staining were quantified and expressed graphically. WT, 73.6% with lamellipodia and 26.4% with stress fibers (n=205); $Ric^{-/-}$, 18.4% with lamellipodia and 81.6% with stress fibers (n=136, $p < 0.0001$ by chi-square test comparing to WT MEF).

Figure 5. **(E)** The percentages of $Ric^{-/-}$ cells transfected with EGFP-PKCz/T560D or EGFP-PKCz/T560A with lamellipodia or stress fibers were quantified and expressed graphically. Note that only the GFP-positive cells (transfected cells) were included in the quantification. EGFP-PKCz/T560D, 63.8% with lamellipodia and 36.2% with stress fibers (n=72); EGFP-PKCz/T560A, 30.4% with lamellipodia and 69.6% with stress fibers (n=79, $p < 0.0001$ by chi-square test comparing between the two groups).

In addition, we found that the patterns of actin organization remained unchanged in untransfected cells. We clarified this in the manuscript (page 10, the last paragraph). Furthermore, we added two more examples for each cell type, including WT, $Ric^{-/-}$, and EGFP-PKCz/T560D or EGFP-PKCz/T560A transfected $Ric^{-/-}$ MEF cells, in the supplemental data. The images of these cells are now shown in Supplemental Figure S2 and Supplemental Figure S4.

2. Both reviewers have suggested to include discussion of PKCz substrate.

To this end, we added reference to LLGL2 as a potential substrate of PKCz that may be involved in regulating cell migration (page 12).

3. The authors should also mention in the discussion that the constitutive nature of phosphorylation of the TM (of Akt and PKC) was previously demonstrated to be due to occurrence of this mTORC2-mediated phosphorylation during translation (see Oh et al, EMBO J 2010), hence could be a similar mechanism for PKC-zeta.

This reference has been cited and briefly discussed (page 11, the last paragraph).

4. They should avoid stating that "PKC-iota is a substrate of mTORC2". They did not directly demonstrate this, they only showed that the TM phosphorylation of PKC-iota is sensitive to mTOR inhibitors.

This has been corrected according to reviewer's suggestion.

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5. I might also bring the following paper to the attention of both you and the authors; I don't believe it is referenced but it is certainly relevant and might affect impact.
Cancer Res. 2010 Nov 15;70(22):9360-70. mTOR complex component Rictor interacts with PKCzeta and regulates cancer cell metastasis.
Zhang F, Zhang X, Li M, Chen P, Zhang B, Guo H, Cao W, Wei X, Cao X, Hao X, Zhang N.

This reference has been cited and briefly discussed (page 12).

6. I have noted that you need to expand the Materials and Methods section.

The Materials and Methods section has been expanded.

Additional correspondence (editor) 09 October 2013

Thank you for the submission your revised study to EMBO reports. Unfortunately, some problems have come up during our routine data check prior to making a final decision.

Figures 1C and 4B seem to have been subject to inappropriate image processing. Importantly, the lanes in the upper blot of figure 2C (Flag-IP p410) denoting vector and 410E with and without insulin, appear to have been duplicated. This type of alteration would prevent the publication of your study in EMBO reports.

I am sorry that these issues have come up, but we cannot proceed with publication under these circumstances.

Additional correspondence (author) 11 October 2013

Thank you for giving us an opportunity to clarify issues related to figures in our manuscript. I apologize for mistakes and lack of complete understanding of the guidelines when preparing the manuscript. In the following “Specific Responses” section, please find our responses in order to correct mistakes in each figure in question. The revised figures with source files are attached as individual TIF files.

Specific Responses:

For Figure 1C, we attached two source files named “Fig 1C-mTOR, rictor, raptor in IP and lysates” and “Fig 1C-PKC in IP and lysates”. Annotations were added on the edges of images (outside the real image areas) for easier orientation. After reading the Instruction to Authors from EMBO Reports, I realized that we did not follow the guideline when presenting cropped images. We should leave a boundary between the two parts of the image once cropped. To correct this, Figure 1C now presented the cropped images as two separate panels according to the guideline. I attached the revised Figure 1 for your review. Please check the new figure against the source files.
For Figure 2C, five source files are attached for the five image panels shown in the figure (named as Fig 2C- followed by the name of the panel). I also attached the revised Figure 2 in which the panel in question (Figure 2C, Flag IP-p410 panel) was replaced. Please check against the source files.

For Figure 4B, the only source file that I could find is the same image as included in the figure. As I mentioned to you in one of our earlier communications, the postodoc who worked on this project had left the lab. During the manuscript revision process, I personally repeated all the immunofluorescence staining experiments, and I took dozens more images of both WT and Rictor null MEF cells. In fact, the additional images shown in Supplemental Figures S2 and S3 were taken from the experiments that I did recently. Since the quantification results shown in Figures 4 and 5 included images from the new experiments, to ensure the accuracy and to eliminate any possible bad

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processing of Figure 4B, I felt the best way is to replace it with an image that I took in recent experiments. I attached the revised Figure 4 and the source image for the new Figure 4B for your review.
Not to find excuses, but we do use a shared microscope facility where images files are purged from microscope-associated computers regularly. After searching through hundreds of files, it seemed that only processed images from previous experiments are saved and transferred to our lab computers. It is likely that mistakes were introduced when the scale bar was added. Perhaps the position of one area of the image got moved but not the rest of the image. However, I had no doubt on the image itself in term of its scientific representation. We have many more images of Rictor null MEF cells (including images that I obtained myself) showing the same distribution pattern of actin. The quantified results demonstrate this as well. Again, I apologize for overlooking any processing mistakes. Hopefully, the revised figures address the concerns.

3rd Editorial Decision 22 October 2013

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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