Analysis of the TORC1 interactome reveals spatially distinct function of TORC1 in mRNP complexes

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Scientific Editor: Andrea Marat

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January 29, 2020

Re: JCB manuscript #201912060

Dr. Won-Ki Huh
Seoul National University
School of Biological Sciences
Seoul National University
Seoul 08826
Korea (South), Republic of

Dear Dr. Huh,

Thank you for submitting your manuscript entitled "Analysis of TORC1 interactome reveals spatially distinct function of TORC1 in mRNP complexes". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that while the reviewers find that your study represents a potentially important advance, several key points need to be strengthened. In particular, to resubmit to JCB it is essential to address the concern of Reviewer #1 (Major concern 1) to use a more appropriate promoter without huge overexpression. However, while further insight into how Scd6- and TORC1-associated mRNAs are being regulated (as discussed in Rev #1's major concern 2) is certainly an important research avenue, it can be the subject of future studies. Another essential point to address in detail is major point 5 of Reviewer #2, to provide more physiological evidence supporting the relationship between TORC1 and Scd6. In addition, we hope that you will be able to address all of the remaining reviewer comments in your revised manuscript.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

The typical timeframe for revisions is three months; if submitted within this timeframe, novelty will not be reassessed at the final decision. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Johan Auwerx, MD, PHD
Monitoring Editor
Journal of Cell Biology

Andrea L. Marat, PhD
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In the manuscript, Chang et al. are investigating an already proposed link between TORC1 and P-bodies/Stress Granules. Using a split fluorescent protein system to detect protein-protein interactions, they found that a minor fraction of TORC1 interacts with mRNP-associated factors and co-localize with cytosolic P-bodies/stress granules. The authors propose that TORC1 association to p-bodies would regulate, in a Scd6 phosphorylation-dependent manner, the translation of a subset of mRNAs which is a novel and important advance.
Major Concern:
1) The BiFC fails to detect specific interactions between the VC-tagged bait and known interactors. For instance, interactions between TORC1 subunits (between Tor1/Tor2, Kog1 and Lst8) also Ego3 does not show any interaction with other EGO complex subunits (Ego1, Ego2 and Gtr1/2). Additionally, more than 85% of reported Kog1 interactors have not been identified (Figure 1F). On the other hand, it appears that chaperones activated by aggregation and unfolded proteins (HSP104 and HSP26) are systematically interacting with each bait proteins used. This suggests that expression of the VC-tagged proteins may lead to partial protein unfolding as expected for over-expression of individual subunits of multi-protein complexes (TORC1 and EGO complexes). Unfortunately, the promoters used to drive the VC-tagged protein expression (CET1 and HXK2 promoters) lead to approximately 2,500 and 200,000 protein molecules per cell, respectively (Ho B.

et al., 2018) while the endogenous promoters of TORC1 subunits produce about 1,000 protein molecules per cell. Actually, a dose-dependent effect has been clearly highlighted by the data. The co-localisation per cell is multiplied by 2.5 to 3.5 times comparing CET1 to HXK1 promoters (Figure 2F and H). We suspect that the non-endogenous promoters used in this study is inducing non-specific interactions by expressing lonely and unfolded subunits. Strains expressing GFP-TOR1 and GFP-KOG1 under their own promoters have to be used to verify these interactions.

The TORC1 focus has been recently deeply investigated. In exponentially growing cells, TORC1 has been localized diffusely on the vacuole surface (only 10% of cells contain a focus). In contrast, a majority (~80%) of starved cells display a TORC1 focus. Indeed, depleting glutamine or glucose rapidly triggers TORC1 focus formation (eg. Hallett et al., 2015 and Ukai et al., 2018). Strikingly, in the over expressed GFP-Tor1 strains used by the authors, already 80% of cells display foci before glucose deprivation (Figure 3A). The worry is that the high number of cells displaying a focus is an artefact of protein overexpression.

Key experiments should be repeated using endogenous promoters. For example, there is no a priori reason why pull-down experiments should be done with over-expression constructs - tagging of the endogenous genes should be done instead. Without these controls it is not possible to judge how useful the BIFC screen actually is.

2) TORC1-dependent phosphorylation of Scd6 in vivo should be demonstrated. Ideally, all Scd6-dependent regulations observed in the current manuscript should be tested upon acute TORC1 inhibition. How are the Scd6- and TORC1-associated mRNAs being regulated? Stability? Translation? Both or neither? This is the truly novel aspect of this work and should be further developed.

Other requests:
1) A vacuolar marker (ie Vph1) should be used for results dealing with vacuolar localization. Ideally, endosomal markers would be used as well to compare / contrast current localization results with recent work (cited) from the De Virgilio group.
2) Page 3, The Ego Complex also contains Ego2.
3) Page 4, doi: 10.1016/j.cell.2013.01.033. should be cited
4) Any strain expressing a tagged TORC1 subunit should be tested for its sensitivity to rapamycin to assess if the modification perturbs TORC1 activity.
5) Co-localisation of TORC1 complex and P-bodies/Stress Granules has already been reported (Struhl et al., 2015 and Takahara et al. 2012). However, the biological relevance of such localisation remains debated. Especially, the fraction of co-localisation fluctuates from 3% to 30%. In the current paper, it is impossible to assess if the quantification has been done in a correct manner. The authors should describe better the microscopy quantification procedures.
6) Figure 1B - Label that we are observing BIFC interaction with Kog1

Reviewer #2 (Comments to the Authors (Required)):

The manuscript "Analysis of TORC1 interactome reveals spatially distinct function of TORC1 in mRNP complexes" by Chan et al. reports on the TORC1 interactome in yeast. The authors took a genome-wide approach taking advantage of a biomolecular fluorescence complementation assay. They found that in addition to vacuolar proteins, which reside at the main TORC1 localization site, many Kog1 interactors reside at cytoplasmic messenger ribonucleoprotein granules. Thus, they propose mRNP-granules (p-bodies) as a new TORC1 localization site. Furthermore, Chan et al. propose that the newly identified TORC1 substrate Scd6, a translation repressor, is negatively

regulated by TORC1 mediated-phosphorylation at p-bodies.

This manuscript is a valuable contribution to the understanding of TORC1 action at RNA granules and will represent a considerable advance to the field, subject to the following revisions:

Detailed feedback

Major points:

1. The author state (Line 105-108) "that Kog1 is a defining component of TORC1" and thus, they propose "that 130 Kog1 interactors could represent [the] TORC1 interactome". However, only 37 (see Figure 1C) of these proteins overlap with either the Tor1 or the Tor2 interactomes. It is unclear to the reviewers how the authors explain this discrepancy.

a. How many of the 28 Kog1 interactors validated by co-IP (Figure 1D) were also identified in the Tor1 and Tor2 BiFC assay?

b. The reviewers suggest that (1) either only components are considered as TORC1 specific, which also interact with Tor1 and/or Tor2 or (2) the authors clearly state that the identified components are Kog1 and not TORC1 specific.

c. In addition, for Figure 1D, the reviewers suggest to provide immunoblot detections for all 28 validated interactors. Also the immunoblot data for the non-validated interactors should be added to the supplements.

2. The authors claim that their BiFC data is highly complementary to the existing data sets in SGD, as they identified 121 novel Kog1 interactors. However, the reviewers wonder why out of 130 proteins only 9 overlap with the known 78 Kog1 interactors previously published and annotated in the SGD database. Can the authors further explain this low overlap, and why so many known Kog1 interactors were not identified in their BiFC approach.

3. In Figure 4 and S4, the authors do not mention the number of replicates for the kinase assays. Please include this information.

Also add quantifications and statistics across the replicates for the experiments shown in Figure 4 and the data in 3F, 5A, and S3.

4. The authors use a mutagenesis approach to show that the interaction between Scd6 and Dhh1 is modulated by Scd6's phosphorylation status (Figure 5A and B). Rapamycin strongly reduces the phosphorylation of Scd6 (Figure 4C). The reviewers wonder if pharmacological TORC1 inhibition is also having an effect on the Scd6 - Dhh1 interaction. Thus, we suggest to include the analysis of the interaction between Scd6 and Dhh1 by co-IP assays with and without rapamycin pre-treatment of the cells.

5. The authors comment on TORC1 phosphorylation of Scd6 at p-bodies as a mechanism for translation de-repression, and they use Ego1-fusion constructs to show that the susceptibility to Scd6 overexpression relates to the localization of TORC1 to or away from the vacuole (Figure 5E,F).

a. Can this observation be recapitulated by a more physiological setup that relocalizes TORC1 to or from the vacuole, e.g. nutrient starvation vs. sufficiency? Would for instance the comparison of glucose or nitrogen starvation vs. sufficiency show a similar difference in rescuing/aggravating the cell growth phenotype upon Scd6 overexpression? This seems to be indicated by data shown in Figure 5F and S5D, but should be analyzed side by side.

b. In addition, is the TORC1-dependent Scd6 phosphorylation enhanced by nutrient starvation, as suggested by co-localization of p-bodies with TORC1 upon glucose starvation (Figure 3A,B)?

6. In Figure 6 and associated supplementary figure, the authors claim the specific enrichment of mRNA species in Kog1 IPs. Yet, transcripts highly expressed in the cell may accumulate non-selectively. This cannot be ruled out by normalizing the enrichment to the input (Figure 6B) or by showing that the transcripts are not enriched in an Sch6 IP (Figure S6A, B). The reviewers suggest to include the relative expression of the candidate transcripts within the transcriptome.

Minor points:
1. The scheme displayed in Figure 1G is not clear.
Please provide a list of proteins identified for each GO term.
In addition, please specify the meaning of "background".

2. Figure 3F: please include the treatment scheme, in which Myc- or GFP- tagged proteins were over-expressed.

3. Please provide better quality data for the immunoblots shown in Figure 4B-D for FLAG-Kog1 and HA-Tor1.
For FLAG-Kog1 the specific signal is barely visible (Figure 4B).
For HA-Tor1 the contrast is too high, so it cannot be assed if there is a signal in some of the lanes (i.e. lane 1 and 2, Figure 4C; lane 1 and 3 in Figure 4D).

4. In Supplementary Figure 4B and C, the authors performed similar experiments as in Figure 4. However, the blots in which they detected HA-Tor1 and Kog1 look different. Please indicate which methodological difference leads to this difference in appearance.

5. In line 77-79 the author state "We show that non-vacuolar TORC1 has interaction with specific mRNAs and three mRNA-binding proteins among TORC1 interactome, i.e., Scd6, Ssd1, and Whi3, are phosphorylated by TORC1." Of note, Ssd1 was previously found in genetic screens to interact with TOR1 and TOR2 (PMID: 22296835 and 27708008; https://www.yeastgenome.org/locus/S000002701/interaction). Therefore, the authors should describe in their discussion the previous data about Ssd1 in relation with Tor1 and Tor2 and compare them to their findings.

6. The authors should include a reference in line 183, where they introduce liquid-liquid phase separation in mRNA granules.

7. In lines 37 - 43 the authors claim "TORC1 regulates temporal aspects of cell growth (…). TORC2 regulates spatial aspects of cell growth by controlling the organization of the actin cytoskeleton (Loewith et al., 2002; Wullschleger et al., 2006)." This view is somewhat outdated as ample evidence has accumulated since 2006 that TORC2 is an important metabolic controller as well. See for instance the review of Roelants et al (2017) from the Thorner lab (PMID: 28872598). Please revise the statement based on the newer literature.
Revise also throughout to recognize the recent literature.

8. Cite also Wippich et al (PMID: 23415227) for the statement in lines 57 - 61.

9. Please correct the typo in line 121 "throughput format and out data set is highly complementary to the existing data sets". Replace "out" with "our".
The article is missing in several instances, e.g. in line 44.

Please revise grammar and typos carefully throughout.

Kathrin Thedieck & Alexander Martin Heberle, Marti Cadena Sandoval, Ulrike Rehbein, and Jose Ramos Pittol (Lab for Metabolic Signaling, Innsbruck University)

Response to the comments of Reviewer #1.

In the manuscript, Chang et al. are investigating an already proposed link between TORC1 and P-bodies/Stress Granules. Using a split fluorescent protein system to detect protein-protein interactions, they found that a minor fraction of TORC1 interacts with mRNP-associated factors and co-localize with cytosolic P-bodies/stress granules. The authors propose that TORC1 association to p-bodies would regulate, in a Scd6 phosphorylation-dependent manner, the translation of a subset of mRNAs which is a novel and important advance.

➔ We thank the reviewer for his/her thoughtful and perceptive comments.

Major Concern:

1) The BiFC fails to detect specific interactions between the VC-tagged bait and known interactors. For instance, interactions between TORC1 subunits (between Tor1/Tor2, Kog1 and Lst8) also Ego3 does not show any interaction with other EGO complex subunits (Ego1, Ego2 and Gtr1/2). Additionally, more than 85% of reported Kog1 interactors have not been identified (Figure 1F). On the other hand, it appears that chaperones activated by aggregation and unfolded proteins (HSP104 and HSP26) are systematically interacting with each bait proteins used. This suggests that expression of the VC-tagged proteins may lead to partial protein unfolding as expected for over-expression of individual subunits of multi-protein complexes (TORC1 and EGO complexes). Unfortunately, the promoters used to drive the VC-tagged protein expression (CET1 and HXK2 promoters) lead to approximately 2,500 and 200,000 protein molecules per cell, respectively (Ho B. et al., 2018) while the endogenous promoters of TORC1 subunits produce about 1,000 protein molecules per cell. Actually, a dose-dependent effect has been clearly highlighted by the data. The co-localisation per cell is multiplied by 2.5 to 3.5 times comparing CET1 to HXK1 promoters (Figure 2F and H). We suspect that the non-endogenous promoters used in this study is inducing non-specific interactions by expressing lonely and unfolded subunits. Strains expressing GFP-TOR1 and GFP-KOG1 under their own promoters have to be used to verify these interactions.
The TORC1 focus has been recently deeply investigated. In exponentially growing cells, TORC1 has been localized diffusely on the vacuole surface (only 10% of cells contain a focus). In contrast, a majority (~80%) of starved cells display a TORC1 focus. Indeed, depleting glutamine or glucose rapidly triggers TORC1 focus formation (eg. Hallett et al., 2015 and Ukai et al., 2018). Strikingly, in the over expressed GFP-Tor1 strains used by the authors, already 80% of cells display foci before glucose deprivation (Figure 3A). The worry is that the high number of cells displaying a focus is an artefact of protein overexpression.
Key experiments should be repeated using endogenous promoters. For example, there is no a priori reason why pull-down experiments should be done with over-expression constructs - tagging of the endogenous genes should be done instead. Without these controls it is not possible to judge how useful the BIFC screen actually is.

➔ The reviewer pointed out potential risk of using overexpression system and suggested to use the native promoters for interaction analysis. The reviewer suspected that (1) failure in detection of known interactors of TORC1, (2) interaction with chaperones, and (3) high percentage of foci-displaying cells may result from side effects of overexpression system.

A. The *HXK2* and *CET1* promoters
First, we would like to explain about the expression system that we used. Endogenous chromosomal C-terminal tagging is a convenient way for expressing tag fusion proteins under the control of native promoters. However, because C-terminal tagging of Tor1 and Kog1 causes severe defects in their function (see Fig. R4), we thought that it is not proper to use C-terminal tagging system for Tor1 or Kog1. Instead, we tried several times to achieve endogenous chromosomal N-terminal tagging of Tor1 and Kog1 with their own promoters, but we failed to generate appropriate strains probably due to higher recombination at undesirable sites. Endogenous chromosomal N-terminal tagging with the native promoter is technically challenging (Booher and Kaiser, 2008), and thus it is usually conducted by insertion of a heterologous promoter with tag at the upstream of target gene. Therefore, we decided to use a heterologous promoter that has similar expression strength to those of *TOR1* and *KOG1*.
The *CET1* promoter is one of the modular promoters that our lab has already constructed and confirmed the utility. According to (Ho et al., 2018) paper, protein levels of *CET1* is 2.8- and 2.1-fold higher than those of *TOR1* and *KOG1*, respectively. However, because protein abundance can be affected by not only transcription/translation but also protein stability, we believe that transcript levels are more suitable for comparing the strengths of promoters. From duplicated RNA-seq data, we found that the FPKM values of *TOR1* were 20.9/22.3 and those of *KOG1* were 23.4/22.3, which were not significantly different from the FPKM values of *CET1* (26.1/27.3). As reviewers noted, the FPKM values of *HXK2* were much higher (1119.6/1138.9) than those of *TOR1* or *KOG1*. We also confirmed this result with qRT-PCR and Western

blotting. As shown below, mRNA expression levels of *TOR1* and *KOG1* under the *CET1* promoter were comparable to those of *TOR1* and *KOG1* under the native promoters (Fig. R1A; this data has now been included in the revised manuscript as Fig. S3A). Consistent with this, protein levels of GFP-Kog1 expressed under the *CET1* promoter were similar to those of Kog1-GFP expressed under the native promoter (Fig. R1B; this data has now been included in the revised manuscript as Fig. S3B).
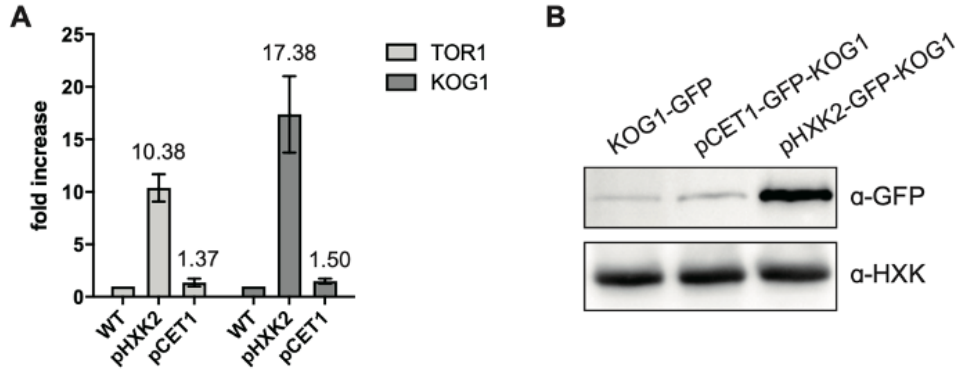

Fig. R1. (A) mRNA levels of *TOR1* and *KOG1* under the native, *HXK2*, and *CET1* promoters. C_T values were normalized to those of *ACT1*. (B) Protein levels of Kog1 under the native, *CET1*, and *HXK2* promoters.

Given that the *CET1* promoter exhibits comparable activity with the native promoters of *TOR1* and *KOG1*, we performed most of biochemical works using the *CET1* promoter. Especially, all of Fig. 3 (except 3A), 4, 5, Fig. S3C-E, S4 were obtained using GFP- or HA-tagged Tor1/Kog1 expressed under the *CET1* promoter. In addition, we further examined whether 28 interactors of *prHXK2-GFP-KOG1* exhibit co-IP-positive signals with *prCET1-GFP-KOG1*. We observed that 18 out of 28 proteins showed co-IP-positive signals with *prCET1-GFP-KOG1* (Fig. R2). Although, unfortunately, some of 28 interactions were not detected under our condition, this result suggests that Kog1 interactors we found are not all artefacts derived from overexpression.
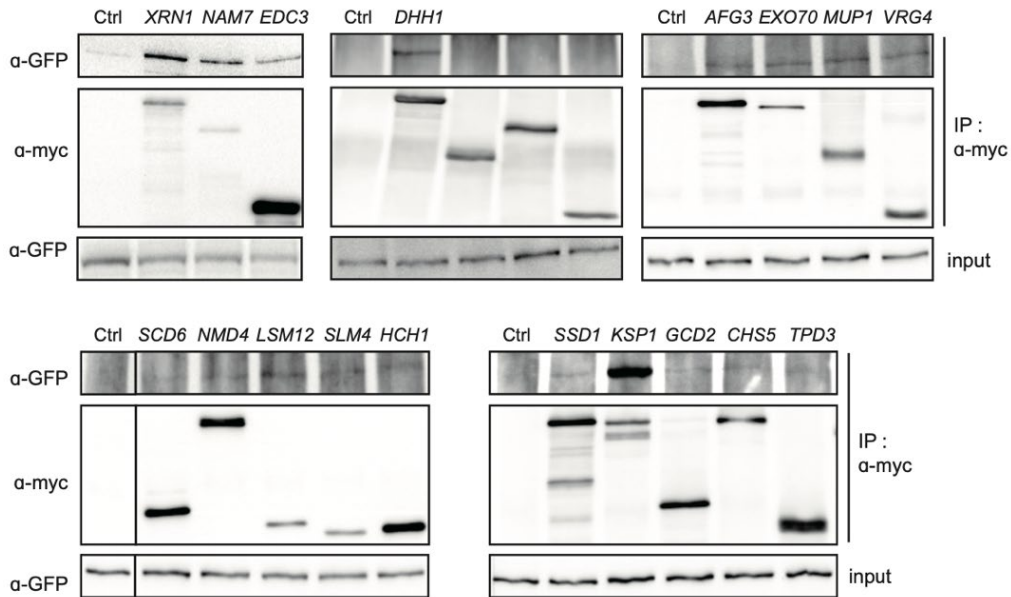

Fig. R2. Co-IP assay for Kog1 interactors. GFP-Kog1 was expressed under the *CET1* promoter and Myc-tagged proteins were expressed under their native promoters.

It was also one of our major concerns to avoid artefacts derived from overexpression while taking advantage of overexpression system in high-throughput analysis and imaging. We note again that the *HXK2* promoter was only used to find potential interactors and location of TORC1 throughout this paper. In addition, when we used overexpression system, we expressed both Tor1 and Kog1 under the *HXK2* promoter to minimize the artefacts caused by lonely subunits. Other experiments for functional study of TORC1 were conducted using the *CET1* promoter.

B. Failure in detection of known interactors of TORC1
① Interaction with EGO complex
Interaction between TORC1 and EGO complex has been validated in many studies (Binda et al., 2009; Bonfils et al., 2012; Sekiguchi et al., 2014; Ukai et al., 2018). We also detected BiFC signals between EGO complex and TORC1. We summarize the results in the table below.

	Meh1-VN (Ego1-VN)	Ego2-VN	Slm4-VN (Ego3-VN)	Gtr1-VN	Gtr2-VN
VC-Tor1	O	Not present in the VN fusion library		O	O
VC-Tor2	O			O	
VC-Kog1	O		O	O	O
VC	O				

In our BiFC results, Ego1 showed positive BiFC signals with all three Tor1, Tor2, and Kog1, but it was eliminated from TORC1 interactome because it also showed positive BiFC signals with VC fragment alone. We note that this validation approach could exclude some true interactions like Ego1. Nevertheless, we excluded all proteins that showed positive signals with VC fragment alone to minimize false-positive candidates as we described in the manuscript.
Other components except Ego2 selectively showed positive signals with at least one of TORC1 subunits. Ego3 was included in Kog1 interactome, and Gtr1 was listed in all the Tor1/Tor2/Kog1 interactomes. Gtr2 showed positive signals with both Tor1 and Kog1. Although the VN fusion library covers ~95% of all yeast ORFs, some genes are missing from the library due to failure during construction. *EGO2* is one of those missing genes, and thus we could not test interaction between Ego2 and TORC1.

② Interaction between TORC1 subunits
As the reviewer pointed out, we could not detect BiFC signals between TORC1 subunits. Regarding this, we suggest three possibilities. i) Topological constraints: Topological constraints are regarded as the major reason for false-negative results of the BiFC assay. Because VN and VC fragments should get close (< 10 nm) to reconstitute a fluorescent protein, BiFC signals are affected by the distance and direction of VN and VC fragments (Kerppola, 2008). Given that the sizes of our bait proteins (Tor1, Tor2, and Kog1) are quite large (180~220 kDa), we suspect that the BiFC complex formation is not favorable between TORC1 subunits. ii) Expression level: Although the BiFC assay has advantages in detecting interactions of low-abundance proteins compared to other PPI techniques (Kim et al., 2019), the average expression levels of BiFC-positive interactors were higher than those of whole proteome (Fig. R3). The endogenous expression levels of TORC1 subunits are lower than those of 90% of BiFC-positive interactors. It is probable that low abundance of TORC1 subunits may make it hard to detect BiFC signals between TORC1 subunits. iii) Functional impairment by C-terminal tagging: We found that C-terminal tagging to Tor1 or Kog1 disrupts their activity (Fig. R4). Because the VN fusion library, which is composed of C-terminally VN-tagged strains, was used in a genome-wide BiFC screen, Tor1-VN and Kog1-VN were included in the BiFC assay. We assume that C-terminally VN-tagged Tor1 or Kog1 may disturb not only the activity but also the integrity of TORC1 and thus hinder proper BiFC complex formation between TORC1 subunits.
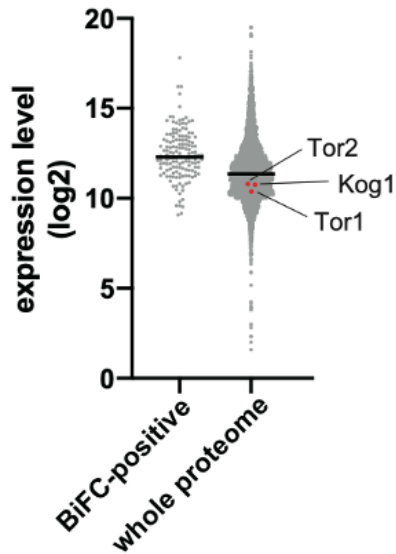
Fig. R3. Protein expression levels are based on (Ho et al., 2018). Bar indicates median of each column. Red dots indicate Tor1, Tor2, and Kog1.
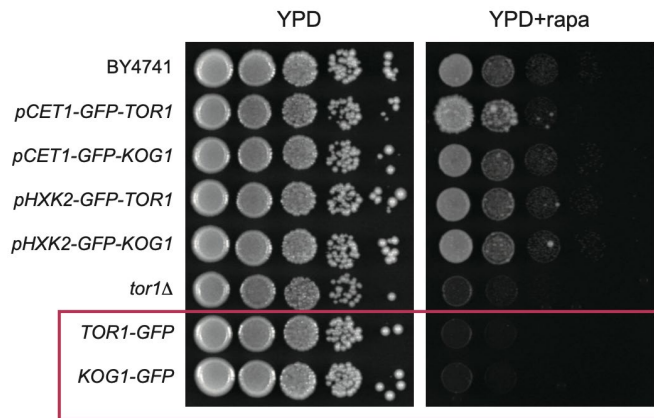

Fig. R4. Impaired TORC1 activity by C-terminal tagging to Tor1 or Kog1.

③ Interaction with known TORC1 interactors
Large-scale interactome analyses generate data sets that depend largely on the employed experimental methods. Thus, it is not unusual that a data set from one study shows only a modest overlap with a data set from other studies. For instance, less than 20% of known SUMO interactors overlap with those identified by a genome-wide BiFC assay (Sung et al., 2013). In the case of protein homomers, among the 186 protein homomers identified in a BiFC screen, 47 overlap with 519 homomer candidates in previous data sets (Kim et al., 2019). This small percentage of overlap is similarly observed in comparisons between other studies (Kim et al., 2019). Given that ~70% of known TORC1 interactors have been identified by affinity capture-based techniques, it is presumable that technical differences contribute to low percentage of overlap between previous data sets and our data set. We also believe that failure in detection of known TORC1 interactors is partly due to the topological constraints in the BiFC complex formation as described above.

C. Interaction with chaperones
Because BiFC signals were hardly detected when the expression levels of bait proteins were low, it was unavoidable to use overexpression system in the BiFC screen for TORC1 interactome. To address the reviewer's concern, we examined whether the overexpression of TORC1 induces the unfolded protein response (UPR) and activates Hsp26 and Hsp104.

The UPR is known to increase the expression of *HSP26*, *HSP78*, *SSA4*, *KAR2*, *LHS1*, and *HRD1* (Macedo et al., 2018; Patil et al., 2012). We compared their FPKM values using our RNA-seq data from WT and TORC1 overexpression strains. Overall, the expression levels of the UPR genes were not significantly altered by TORC1 overexpression, except that the expression of *HSP26* was increased about two-fold in TORC1 overexpression strain (Fig. R5). However, given that TORC1 is known to stabilize *HSP26* mRNAs (Talarek et al., 2010), this increase could be due to elevated TORC1 activity by overexpression.
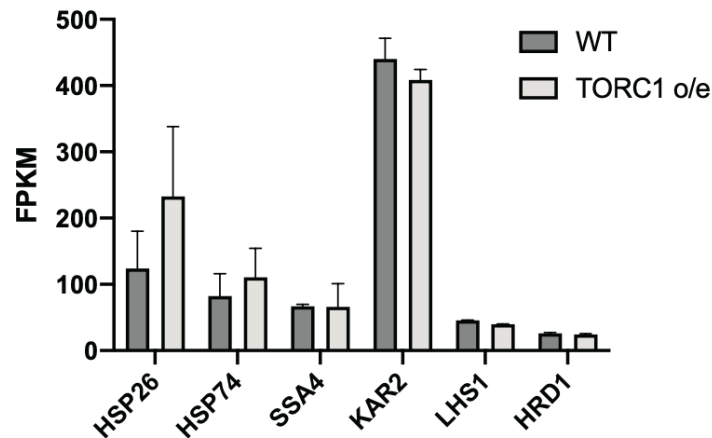

Fig. R5. Expression of UPR genes. C_T values were normalized to those of *ACT1*.

Hsp26 forms cytoplasmic foci in response to heat stress, sequestering misfolded proteins for cellular protection (Ungelenk et al., 2016). Hsp104 also binds to amorphous protein clusters and forms foci wherein Hsp104 rescues aggregated proteins (Kaimal et al., 2017; Paoletti et al., 2016). When we checked whether Hsp26 or Hsp104 foci are formed in the strains that we used in the BiFC screen for TORC1 interactome, we could not observe Hsp26 or Hsp104 foci (Fig. R6). This result suggests that there is little, if any, misfolded protein aggregation in TORC1 overexpression strains.
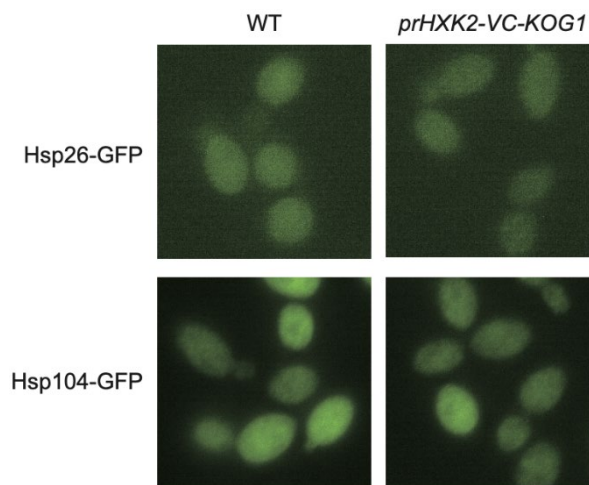

Fig. R6. Examination of Hsp26 or Hsp104 foci formation in cells overexpressing Kog1.

D. High percentage of foci-displaying cells in Fig. 3B.
As we described in Fig. 2G and H, the colocalization of Tor1 foci with P-bodies was observed in ~37% of cells overexpressing TORC1 components under the *HXK2* promoter. We are sorry for providing insufficient

information about Fig. 3B. The Y-axis of Fig. 3B indicates the percentage of cells with each foci relative to cells showing the colocalization of Tor1 foci with P-bodies at 30 min of glucose starvation, not to total cells. We took time-course images at every 2 min after glucose starvation. For 103 cells that exhibited the colocalization of Tor1 foci and Dcp2 at 30 min of glucose starvation, we analyzed the percentage of cells showing Tor1 or Dcp2 accumulation in the foci at each time point. Thus, Fig. 3B does not indicate that >70% of cells have Tor1 foci at the initial time point. To avoid confusion, we included a more detailed description in the legend of Fig.3B.

We are aware of the possibility of overexpression-mediated colocalization, which the reviewer pointed out. However, our results show that deletion of *XRN1* increased the colocalization between Tor1 foci and P-bodies up to 54% even under endogenous-level expression of TORC1 (Fig. 2G and H). Thus, although the overexpression of TORC1 increases the colocalization of TORC1 with P-bodies, we believe that the colocalization between TORC1 and P-bodies is not just an artefact mediated by TORC1 overexpression.

2) TORC1-dependent phosphorylation of Scd6 in vivo should be demonstrated. Ideally, all Scd6-dependent regulations observed in the current manuscript should be tested upon acute TORC1 inhibition. How are the Scd6- and TORC1-associated mRNAs being regulated? Stability? Translation? Both or neither? This is the truly novel aspect of this work and should be further developed.

➔ We fully agree with the reviewer's opinion. Here are our answers to the reviewer's questions.

A. TORC1-dependent phosphorylation of Scd6 in vivo
To detect *in vivo* phosphorylation of Scd6, we first tried to analyze phosphorylation of Scd6 by mass spectrometry (using both GST-Scd6 and Scd6-HA). Unfortunately, despite several attempts, we could not detect phosphorylated residues. It is not unusual that phosphorylated residues are not detected by mass spectrometry. As an example, the known phosphorylated residues of Scd6 (S163/S165) were also not detected under our experimental conditions. We think technical caveats of mass spectrometry make it hard to detect phosphorylation of S256/280/287. Especially, because S287 is embedded with R residues, it is likely that proper fragments including S287 were not produced by trypsinization. Next, we tried to detect phosphorylated S256/280/287 using Western blotting-based techniques. In some cases, phosphorylation on specific residues can decrease the mobility of protein in SDS-PAGE, resulting in the appearance of shifted bands. Unfortunately, however, we could not detect phosphorylated bands of Scd6 in SDS-PAGE. As such, finding evidence for *in vivo* phosphorylation of Scd6 was technically challenging in this study. Nevertheless, given the effect of non-phosphorylatable mutant *in vivo* and the observation that this effect is reproducible by acute inhibition of TORC1 (see below), we believe that S256/280/287 residues are phosphorylated *in vivo*.

B. Scd6-dependent regulations upon acute TORC1 inhibition
In this paper, we proposed that the function of Scd6 is regulated by the interaction with Dhh1 (Fig. 5A and B). To test if acute inhibition of TORC1 can increase Dhh1-Scd6 interaction as the reviewer suggested, we performed co-IP assay for Dhh1-Scd6 interaction under rapamycin treatment conditions. We observed that acute TORC1 inhibition increased the interaction between them (Fig. R7). This result supports our argument that TORC1-dependent phosphorylation regulates Dhh1-Scd6 interaction. This data has now been included in the revised manuscript as Fig. S5B.
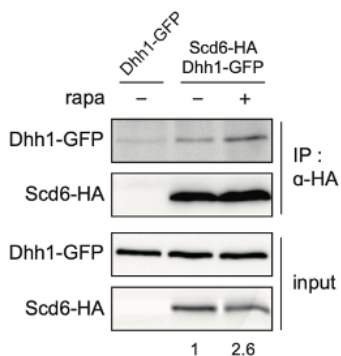

Fig. R7. Interaction between Dhh1 and Scd6 under rapamycin treatment.

Next, we suggested that phosphorylation of Scd6 is important for translational regulation (Fig. 5C). We tried to examine whether this function is also stimulated by TORC1 inhibition. However, because acute inhibition of TORC1 induces global translational repression by well-defined mechanisms, we could not differentiate the effect of dephosphorylated Scd6 from that of TORC1 inhibition.

We also found that non-phosphorylatable Scd6 induces P-body formation at early time-point of glucose starvation (Fig. 5E). To test if acute inhibition of TORC1 also induces P-body formation, we pre-treated cells with rapamycin for 2 hr and monitored P-body accumulation after glucose starvation. We observed the increase in P-body accumulation in rapamycin-treated cells (Fig. R8).
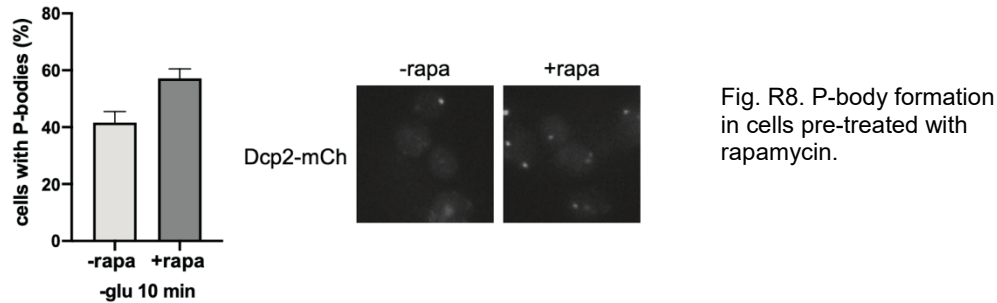

Fig. R8. P-body formation in cells pre-treated with rapamycin.

C. Regulation of Scd6- and TORC1-associated mRNAs

We have identified TORC1-associated mRNAs in this paper and found their overlapping with Scd6-interacting mRNAs. In our preliminary study, we found that the interaction with TORC1 and *ACE2* mRNAs is correlated with *ACE2* expression and overexpression of TORC1 increases cell cycle-dependent expression of *ACE2*. Thus, we hypothesized that TORC1 would enhance the translation of associated mRNAs, which is also in a line with the suggested role of TORC1 in this paper. However, given that *ACE2* is not included in Scd6-associated mRNAs and its regulation is independent of Scd6, it seems that *ACE2* is regulated by another mechanism of TORC1 signaling. We are also investigating a role of TORC1 under heat stress conditions. The expression of TORC1-associated mRNAs tends to increase by heat stress (Gasch et al., 2000) (Fig. R9A). In addition, we found that the heat sensitivity of *tor1Δ* cells was slightly alleviated by deletion of *SCD6* (Fig. R9B), implying a potential role of TORC1-Scd6 axis under heat stress conditions. Now we are trying to find out mRNAs that are targeted by TORC1-Scd6 axis. We fully agree with the reviewer's comment that this finding would be a novel aspect of this paper, but the required experiments are beyond the scope of this paper. We are eager to address this point in our future study.
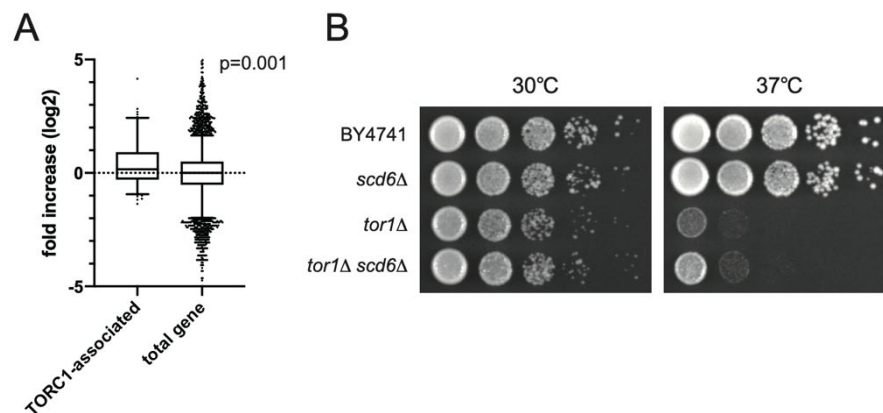

Fig. R9. (A) mRNA expression change under heat stress conditions (29°C → 37°C; Gasch et al., 2000). (B) Heat sensitivity test for the indicated strains. Cells were plated on YPD medium and grown for 2 days.

1) A vacuolar marker (ie Vph1) should be used for results dealing with vacuolar localization. Ideally, endosomal markers would be used as well to compare / contrast current localization results with recent work (cited) from the De Virgilio group.

→ We appreciate the reviewer's comment. We had tested various markers for the vacuolar membrane and the endosome for colocalization analysis, and finally we chose Ego3 among them. Because the location of Ego3 can represent the vacuole as well as paravacuolar foci, we thought that Ego3 is the most appropriate marker for representing "conventional localization of TORC1". To maintain consistency in data presentation, we kept using Ego3 in Fig. 2 in the revised manuscript. Instead, we also performed colocalization analysis using Vph1 as a vacuolar marker or FM4-64 for vacuole/endosome staining to confirm non-vacuolar localization of TORC1 (Fig. R10). This data has now been included in the revised manuscript as Fig. S2D and E.
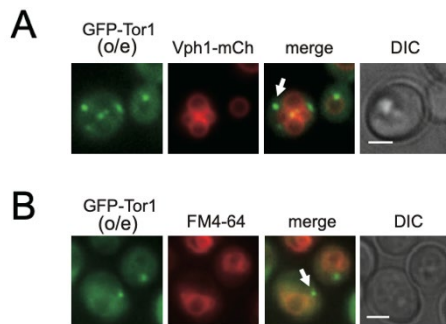

Fig. R10. (A) Colocalization analysis using Vph1 as a vacuolar marker. (B) Colocalization analysis using FM4-64 for vacuole/endosome staining.

2) Page 3, The Ego Complex also contains Ego2.

→ We thank the reviewer for the comment. We included Ego2 in describing the Ego complex and cited a proper reference in the revised manuscript (Lines 46-47).

3) Page 4, doi: 10.1016/j.cell.2013.01.033. should be cited

→ We thank the reviewer for the comment. We cited the paper (Wippich et al., 2013) in the revised manuscript (Line 59).

4) Any strain expressing a tagged TORC1 subunit should be tested for its sensitivity to rapamycin to assess if the modification perturbs TORC1 activity.

→ Because it has been reported that C-terminal tagging to Tor1 inhibits the activity of TORC1, N-terminal tagging is widely used for Tor1 (Adami et al., 2007). We found that C-terminal tagging to Kog1 also severely interferes with the activity of TORC1 (Fig. R4), consistent with a previous report (Berchtold and Walther, 2009). Thus, we used N-terminally tagged Tor1 and Kog1 in this study. This data has now been included in the revised manuscript as Fig. S1A.

YPD   YPD+rapa

BY4741
pCET1-GFP-TOR1
pCET1-GFP-KOG1
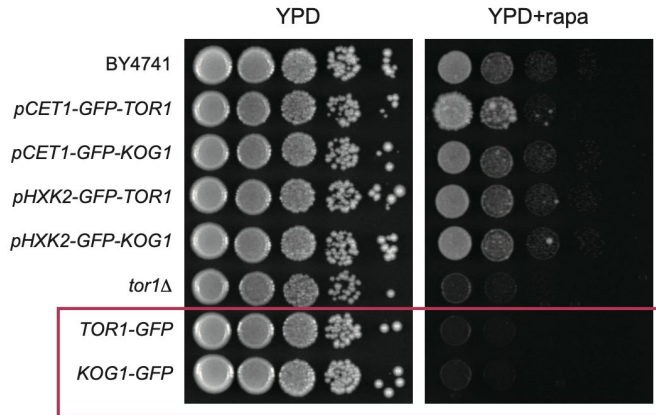pHXK2-GFP-TOR1
pHXK2-GFP-KOG1
tor1Δ
TOR1-GFP
KOG1-GFP

Fig. R4. Impaired TORC1 activity by C-terminal tagging to Tor1 or Kog1.

5) Co-localisation of TORC1 complex and P-bodies/Stress Granules has already been reported (Struhl et al., 2015 and Takahara et al. 2012). However, the biological relevance of such localisation remains debated. Especially, the fraction of co-localisation fluctuates from 3% to 30%. In the current paper, it is impossible to assess if the quantification has been done in a correct manner. The authors should describe better the microscopy quantification procedures.

➔ We appreciate the reviewer's considerate comment. We are sorry but we could not find (Struhl et al., 2015) paper. We guess that the paper the reviewer mentioned is (Hughes Hallett et al., 2015), which reported 3.4% of overlapping between Kog1-YFP and P-bodies, and which Kevin Struhl worked for as a reviewing editor.

As the reviewer pointed out, the colocalization ratios in previous reports fluctuate, which are also different from that in this study. We think the combined effects of differences in experimental, imaging, and analyzing conditions cause the discrepancy of colocalization ratios between this paper and previous reports. (Hughes Hallett et al., 2015) paper reported 3.4% colocalization between Kog1-bodies and P-bodies after glucose starvation for 1 hr. (Takahara and Maeda, 2012) paper reported 30.9% colocalization between TORC1 and stress granules under heat stress at 46°C for 30 min. We also observed that TORC1 is highly colocalized with stress granules as well as P-bodies under the same heat stress condition (Fig. R11). It seems that heat stress condition induces higher colocalization between TORC1 and P-bodies/stress granules than glucose starvation condition.

GFP-Tor1 overexpression   Dcp2-RFP   merge   DIC
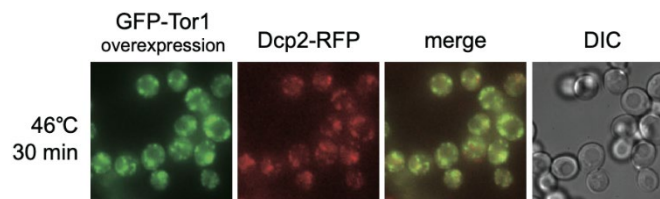
46°C 30 min

Fig. R11. High colocalization between TORC1 and Dcp2 under the same condition of (Takahara and Maeda, 2012).

In (Hughes Hallett et al., 2015) paper, they used Kog1-YFP whereas we used N-terminally GFP-tagged Kog1. We avoided to use C-terminally tagged Kog1 because it causes severe functional defect (Fig. R4). It is presumable that lower expression level of Kog1 also contributed to lower colocalization ratio in (Hughes Hallett et al., 2015) paper.

Our imaging condition is also different from those of previous studies. We used 10 images with 2 μm spacing, whereas (Hughes Hallett et al., 2015) paper used 5 images with 1 μm spacing. Considering that the sizes of mRNP granules are 100~300 nm, we believe that we could detect more granules in cells compared to (Hughes Hallett et al., 2015) paper. In addition, we used ImageJ software to analyze P-bodies. We detected foci signals of Dcp2 above a certain threshold intensity in each image, and then counted the foci that overlapped with TORC1 signals. The threshold for image analysis can be varied among

researchers. It is likely that different threshold is one of the reasons why our colocalization ratio is higher than (Hughes Hallett et al., 2015) paper. We added a detailed description of image analysis in the revised manuscript (Lines 475-482).

6) Figure 1B - Label that we are observing BIFC interaction with Kog1

➜ As suggested by the reviewer, we modified the labels of Fig. 1B and Fig. S2A in the revised manuscript as shown below.

Figure 1B → Revised Figure 1B

Figure S1B → Revised Figure S2A
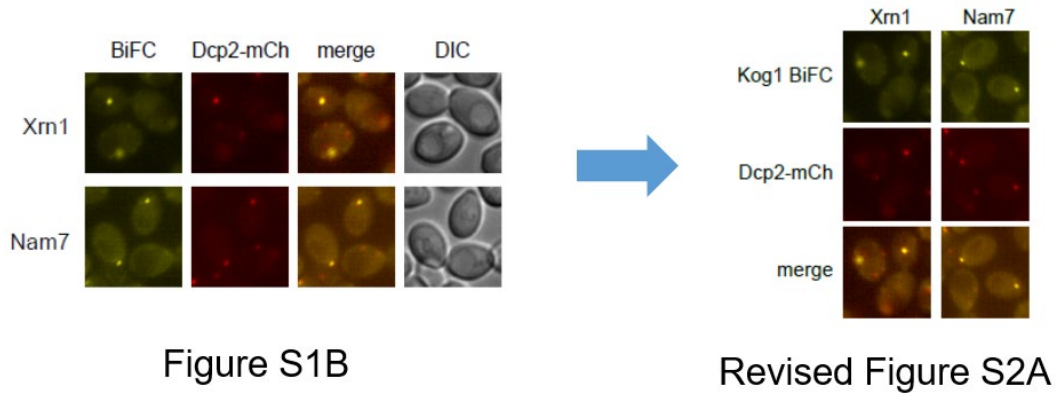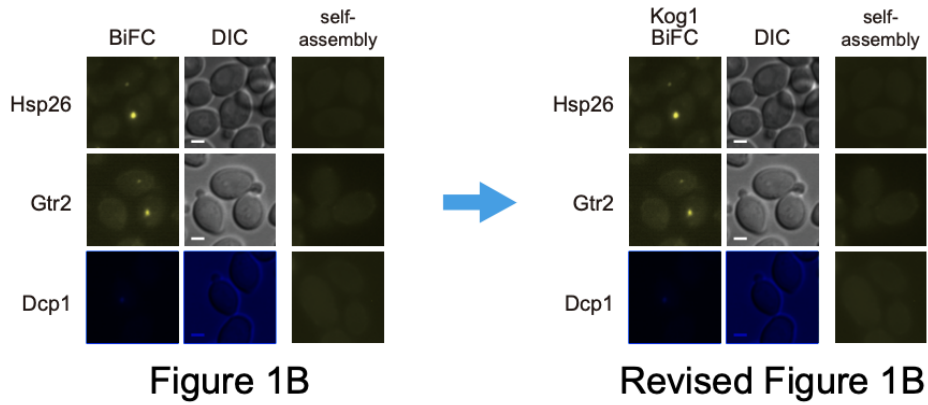
Response to the comments of Reviewer #2

The manuscript "Analysis of TORC1 interactome reveals spatially distinct function of TORC1 in mRNP complexes" by Chan et al. reports on the TORC1 interactome in yeast. The authors took a genome-wide approach taking advantage of a biomolecular fluorescence complementation assay. They found that in addition to vacuolar proteins, which reside at the main TORC1 localization site, many Kog1 interactors reside at cytoplasmic messenger ribonucleoprotein granules. Thus, they propose mRNP-granules (p-bodies) as a new TORC1 localization site. Furthermore, Chan et al. propose that the newly identified TORC1 substrate Scd6, a translation repressor, is negatively regulated by TORC1 mediated-phosphorylation at p-bodies.
This manuscript is a valuable contribution to the understanding of TORC1 action at RNA granules and will represent a considerable advance to the field, subject to the following revisions:

➔ We thank the reviewer for his/her thoughtful and perceptive comments.

Detailed feedback

Major points:

1. The author state (Line 105-108) "that Kog1 is a defining component of TORC1" and thus, they propose "that 130 Kog1 interactors could represent [the] TORC1 interactome". However, only 37 (see Figure 1C) of these proteins overlap with either the Tor1 or the Tor2 interactomes. It is unclear to the reviewers how the authors explain this discrepancy.

➔ We think that the major reason for this discrepancy is the presence of topological constraints in the BiFC complex formation. Because VN and VC fragments should get close (< 10 nm) to reconstitute a fluorescent protein, BiFC signals are affected by the distance and direction of VN and VC fragments (Kerppola, 2008). Given that the sizes of our bait proteins (Tor1, Tor2, and Kog1) are quite large (180~220 kDa), we suspect that the BiFC complex formation with the prey proteins is highly dependent on the topology of Tor1, Tor2, and Kog1. Notably, it has been shown that the N-terminal region of Kog1 is proximal to the kinase domain of Tor1, whereas the N-terminal region of Tor1 is more than 70 Å apart from its kinase domain (Adami et al., 2007).
Another possible explanation for the discrepancy is that Kog1 may have a higher affinity for TORC1 interactors than Tor1 or Tor2. Kog1 is believed to have a role in binding and recruiting the substrates of TORC1 because it has no enzymatic activity but has protein-protein interaction domains (Adami et al., 2007).
Given the limitations of the BiFC assay, we admit that 130 Kog1 interactors do not represent the whole TORC1 interactome. To avoid overstatement, we modified the sentence "we assumed that 130 Kog1 interactors could represent the TORC1 interactome" to "we assumed that 130 Kog1 interactors could represent the majority of the TORC1 interactome".

a. How many of the 28 Kog1 interactors validated by co-IP (Figure 1D) were also identified in the Tor1 and Tor2 BiFC assay?

➔ Among the 28 Kog1 interactors, 5 were detected in both the Tor1 and Tor2 BiFC assay, 6 were detected only in the Tor2 BiFC assay, and 1 was detected only in the Tor1 BiFC assay. The list of overlapping interactors is shown below.

ORF	Gene	Tor1 BiFC	Tor2 BiFC
YOL149W	DCP1	O	O
YNL118C	DCP2	O	O
YMR080C	NAM7	O	O
YML017W	PSP2	O	O
YGL173C	XRN1	O	O
YGR229C	SMI1	O	
YLR330W	CHS5		O
YDL160C	DHH1		O
YLR363C	NMD4		O

YGL252C	*RTG2*		O
YPR129W	*SCD6*		O
YNL197C	*WHI3*		O

b. The reviewers suggest that (1) either only components are considered as TORC1 specific, which also interact with Tor1 and/or Tor2 or (2) the authors clearly state that the identified components are Kog1 and not TORC1 specific.

➔ Kog1 is a defining component of TORC1 in that Tor1 is redundant with Tor2 in TORC1, and Tor2 and Lst8 are also the subunits of TORC2. Only Kog1 is found in TORC1. Thus, it is widely accepted that the interaction or localization of Kog1 is the properties of TORC1 (Binda et al., 2009; Kira et al., 2014; Laxman and Tu, 2011; Sullivan et al., 2019). Nonetheless, given the limitations of the BiFC assay, we admit that 130 Kog1 interactors do not represent the whole TORC1 interactome. As suggested by the reviewer, we used "130 Kog1 interactors" instead of 'the TORC1 interactome", where appropriate, to avoid confusion (Lines 109, 126, 148).

c. In addition, for Figure 1D, the reviewers suggest to provide immunoblot detections for all 28 validated interactors. Also the immunoblot data for the non-validated interactors should be added to the supplements.

➔ As suggested by the reviewer, we included all 28 validated interactors in Fig. 1D and Figure S1C. However, because the raw immunoblot data are composed of 30~40 pictures, we think that including all negative data would be too messy and inefficient. We hope that the reviewer will understand our thought.

2. The authors claim that their BiFC data is highly complementary to the existing data sets in SGD, as they identified 121 novel Kog1 interactors. However, the reviewers wonder why out of 130 proteins only 9 overlap with the known 78 Kog1 interactors previously published and annotated in the SGD database. Can the authors further explain this low overlap, and why so many known Kog1 interactors were not identified in their BiFC approach.

➔ Large-scale interactome analyses generate data sets that depend largely on the employed experimental methods. Thus, it is not unusual that a data set from one study shows only a modest overlap with a data set from other studies. For instance, less than 20% of known SUMO interactors overlap with those identified by a genome-wide BiFC assay (Sung et al., 2013). In the case of protein homomers, among the 186 protein homomers identified in a BiFC screen, 47 overlap with 519 homomer candidates in previous data sets (Kim et al., 2019). This small percentage of overlap (1~7.4%) is similarly observed in comparisons between other studies (Kim et al., 2019). Given that ~70% of known TORC1 interactors have been identified by affinity capture-based techniques, it is presumable that technical differences contribute to low percentage of overlap between previous data sets and our data set. In addition, we also believe that failure in detection of known Kog1 interactors may be due to the following reasons.
① Topological constraints in the BiFC complex formation
Because VN and VC fragments should get close (< 10 nm) to reconstitute a fluorescent protein, BiFC signals are affected by the distance and direction of VN and VC fragments (Kerppola, 2008). The presence of topological constraints in the BiFC complex formation often generate false-negative results for known interactors. Because TORC1 is a quite large complex, the BiFC complex formation with TORC1 would not be favorable for some proteins, depending on their size and the direction of interaction.
② Incompleteness of previous data set
Previous high-throughput experiments to find TORC1 interactors have several caveats. First, TORC1 is unstable in cell lysates (Kim et al., 2002), which makes it hard to detect TORC1 interactors in lysate-based experiments (e.g., affinity purification-WB or -MS). Second, because C-terminal tagging to Tor1 or Kog1 impairs the function of TORC1 (see Fig. R4), previous studies using C-terminally tagged Tor1 or Kog1 would not have led to proper interaction between TORC1 and its interactors. Third, regardless of the employed experimental methods, low expression levels of Tor1 and Kog1 would make it hard to detect positive signals for interaction between TORC1 and its interactors. Given these caveats, it is likely that there are more TORC1 interactors that have not been identified yet.
In this paper, we performed co-IP assay to validate 130 Kog1 interactors identified by the BiFC assay. Among 28 co-IP-positive Kog1 interactors, 24 were included in 121 proteins newly defined by the BiFC assay (Fig. 1F). Given this, we believe that our data set is highly complementary to the existing data sets.

3. In Figure 4 and S4, the authors do not mention the number of replicates for the kinase assays. Please include this information. Also add quantifications and statistics across the replicates for the experiments shown in Figure 4 and the data in 3F, 5A, and S3.

➔ As suggested by the reviewer, we mentioned the number of the kinase assays in the corresponding figure legends in the revised manuscript (Lines 782-784). We also added quantifications and statistics in Fig. 4 (see below). Each value is the mean ± SD of triplicates. In the cases of co-immunoprecipitation data (Fig. 3F, 5A, 5B, S3), although the tendencies of data were consistent between replicates, the values were highly varied depending on experimental conditions (incubation time, antibody/bead concentration, etc.). Thus, we displayed representative data with quantifications for these figures.

Figure 4B

Revised Figure 4B

4. The authors use a mutagenesis approach to show that the interaction between Scd6 and Dhh1 is modulated by Scd6's phosphorylation status (Figure 5A and B). Rapamycin strongly reduces the phosphorylation of Scd6 (Figure 4C). The reviewers wonder if pharmacological TORC1 inhibition is also having an effect on the Scd6 - Dhh1 interaction. Thus, we suggest to include the analysis of the interaction between Scd6 and Dhh1 by co-IP assays with and without rapamycin pre-treatment of the cells.

➔ As suggested by the reviewer, we examined in vivo interaction between Scd6 and Dhh1 under rapamycin treatment conditions. Correlating with our arguments, the Scd6-Dhh1 interaction was 2.6-fold increased by rapamycin (Fig. R12; this data has now been included in the revised manuscript as Fig. S5B).
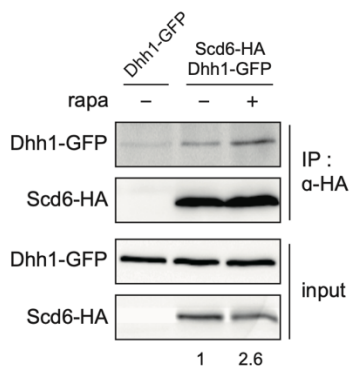

Fig. R12. Interaction between Scd6 and Dhh1 upon rapamycin treatment.

5. The authors comment on TORC1 phosphorylation of Scd6 at p-bodies as a mechanism for translation de-repression, and they use Ego1-fusion constructs to show that the susceptibility to Scd6 overexpression relates to the localization of TORC1 to or away from the vacuole (Figure 5E,F).
a. Can this observation be recapitulated by a more physiological setup that relocalizes TORC1 to or from the vacuole, e.g. nutrient starvation vs. sufficiency? Would for instance the comparison of glucose or nitrogen starvation vs. sufficiency show a similar difference in rescuing/aggravating the cell growth phenotype upon Scd6 overexpression? This seems to be indicated by data shown in Figure 5F and S5D, but should be analyzed side by side.

➔ The reviewer suggested to check the activity of Scd6 when we alter the amount of cytoplasmic TORC1 in more physiological setups. Unfortunately, the conditions for releasing TORC1 from the vacuolar membrane

have not been well identified in yeast. Unlike mammalian TORC1, yeast TORC1 does not leave the vacuolar membrane under starvation conditions. Some previous studies reported the appearance of TORC1 foci under starvation conditions, but the foci still localize to the perivacuolar region (Hughes Hallett et al., 2015). Given a previous report suggesting that there would be a mechanism for dissociating TORC1 from the vacuolar membrane under transient 46°C heat stress (Takahara and Maeda, 2012), we tested the effect of Scd6 overexpression under heat stress conditions. However, 46°C heat stress was so harsh that cells did not grow in our experimental conditions. We exposed cells to a milder heat stress (39°C). However, because heat stress disturbs cellular homeostasis and leads to growth retardation, it was difficult to compare the effect of Scd6 overexpression on cell growth between normal and heat stress conditions. So far, it seems that Ego1 conjugation, which was used in (Takahara and Maeda, 2012) and this study, is the unique way to control the localization of TORC1.

Interestingly, we found that deletion of *TOR1* caused increased sensitivity to heat stress (Fig. R13). This result suggests that the activity of TORC1 is important for maintaining cellular homeostasis under heat stress conditions. Notably, deletion of *SCD6* slightly decreased the heat sensitivity of *tor1Δ* cells. Taken together with a previous report that heat stress may delocalize vacuolar TORC1 (Takahara and Maeda, 2012), it is presumable that delocalized TORC1 may inhibit cell growth via repression of Scd6. This presumption is consistent with our arguments suggested in this paper.
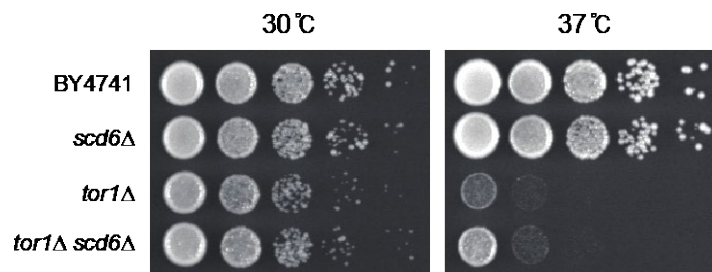

Fig. R13. Heat sensitivity of the indicated strains. Cells were spotted in 10-fold serial dilutions on YPD plates and grown for 2 days.

b. In addition, is the TORC1-dependent Scd6 phosphorylation enhanced by nutrient starvation, as suggested by co-localization of p-bodies with TORC1 upon glucose starvation (Figure 3A,B)?

→ Although Scd6 is a P-body component and P-body-TORC1 colocalization is increased by starvation, overall data in this paper suggest that TORC1 consistently phosphorylates Scd6 under normal condition, preventing recruitment of Dhh1 (Fig. R14). It is known that glucose starvation inhibits TORC1 activity via Snf1 pathway. Thus, we suppose that glucose starvation decreases TORC1-dependent Scd6 phosphorylation, partially contributing to P-body formation. However, because there is no available experimental condition that can detect de novo phosphorylation of Scd6, we could not examine the starvation-dependent phosphorylation of Scd6.
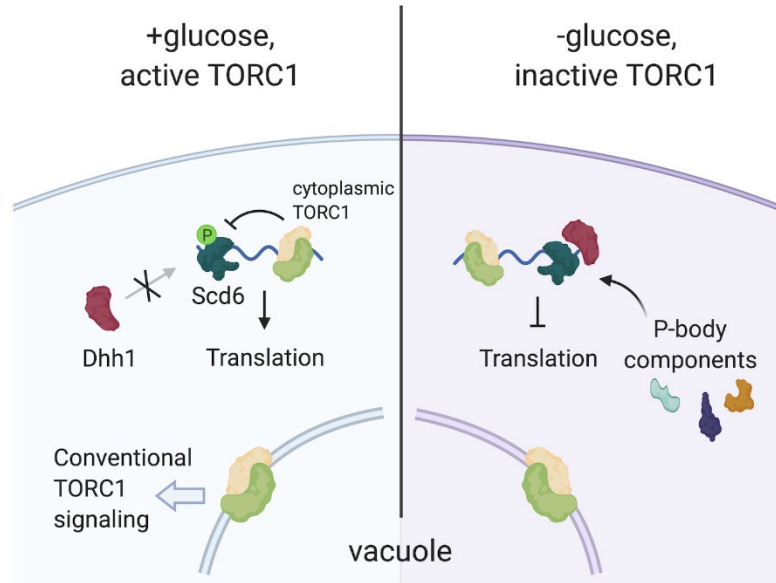
Fig. R14. A proposed model for the regulation of Scd6 by TORC1.

6. In Figure 6 and associated supplementary figure, the authors claim the specific enrichment of mRNA species in Kog1 IPs. Yet, transcripts highly expressed in the cell may accumulate non-selectively. This cannot be ruled out by normalizing the enrichment to the input (Figure 6B) or by showing that the transcripts are not enriched in an Sch6 IP (Figure S6A, B). The reviewers suggest to include the relative expression of the candidate transcripts within the transcriptome.

➔ We totally agree with the reviewer's concern. Based on our RNA-seq data, we compared the FPKM of 120 TORC1-associated mRNAs and total mRNAs. As shown below, there was no bias in expression levels between TORC1-associated mRNAs and total mRNAs (Fig. R15).
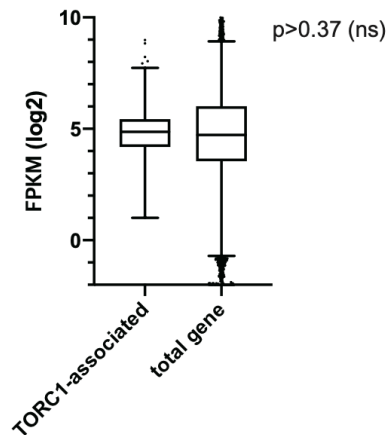

Fig. R15. Distribution of RNA expression levels. The whiskers represent the 5th and the 95th percentiles. *P*-value was calculated by Mann-Whitney *U* test.

Minor points:

1. The scheme displayed in Figure 1G is not clear. Please provide a list of proteins identified for each GO term. In addition, please specify the meaning of "background".

➔ Following the reviewer's suggestion, we provided a list of proteins for each GO term in Supplementary Table 6.

The meaning of "background" is the whole yeast genome in database. To avoid confusion, we replaced "background" with "total gene" in Fig. 1G.
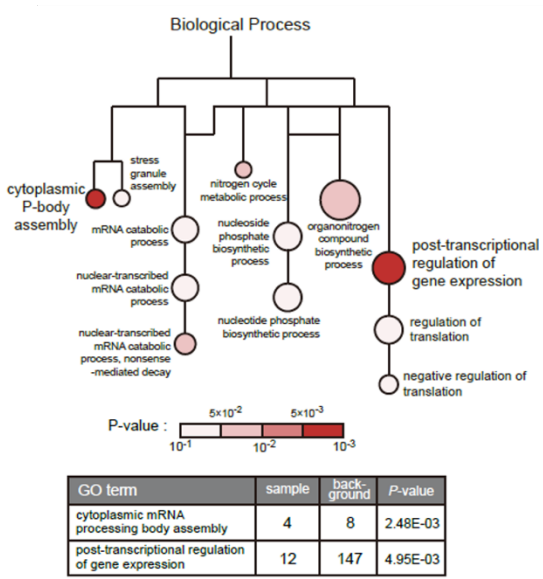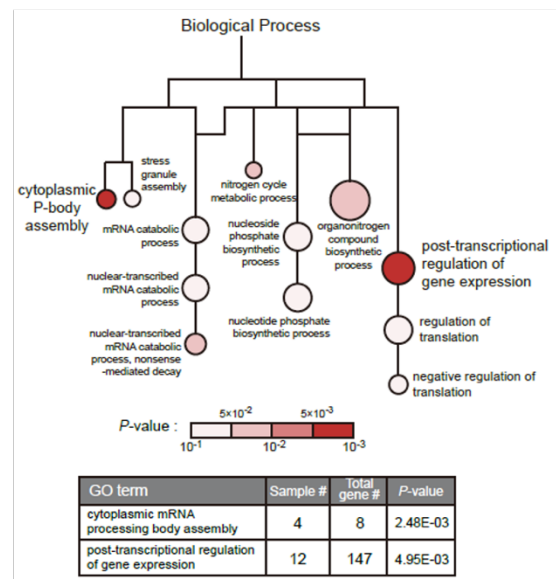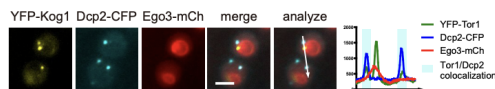

Figure 1G

Revised Figure 1G

2. Figure 3F: please include the treatment scheme, in which Myc- or GFP- tagged proteins were over-expressed.

➔ In Fig. 3F, GFP-Tor1 was expressed under the *CET1* promoter, which has similar strength to that of the *TOR1* promoter (see Fig. R1). Myc-tagged proteins were expressed under their own promoters. As suggested by the reviewer, we included the treatment scheme in the figure legend (Lines 767-769). We also indicated overexpression ("o/e") when proteins were expressed under the *HXK2* promoter, as shown below.
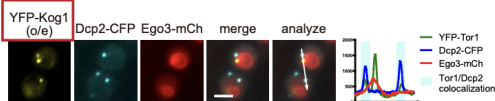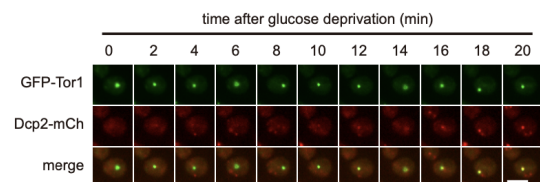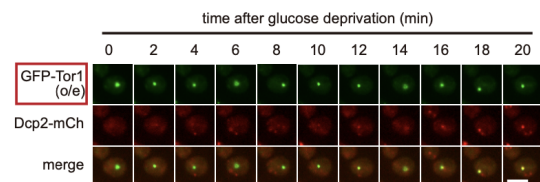

Figure 2D

Revised Figure 2D

Figure 3A

Revised Figure 3A

3. Please provide better quality data for the immunoblots shown in Figure 4B-D for FLAG-Kog1 and HA-Tor1. For

FLAG-Kog1 the specific signal is barely visible (Figure 4B). For HA-Tor1 the contrast is too high, so it cannot be assed if there is a signal in some of the lanes (i.e. lane 1 and 2, Figure 4C; lane 1 and 3 in Figure 4D).

➔ We are sorry for providing insufficient information in the figure legend. FLAG-Kog1 in Fig. 4B was stained with Coomassie blue and thus the bands were faint. To avoid confusion and ensure the unity of data, we replaced the Coomassie blue staining data with the immunoblotting data in Fig. 4B, as shown below. In Fig. 4C and D, although we changed the contrast of HA-Tor1, there was no signal in HA-Tor1-negative lanes.

Figure 4B

Revised Figure 4B

4. In Supplementary Figure 4B and C, the authors performed similar experiments as in Figure 4. However, the blots in which they detected HA-Tor1 and Kog1 look different. Please indicate which methodological difference leads to this difference in appearance.

➔ HA-Tor1 and Kog1 bands in Fig. S4B and C were silver stained. By showing both HA-Tor1 and Kog1, we assured that the integrity of TORC1 was maintained in our experimental conditions. To avoid confusion, we included a description ("Tor1 was immunoprecipitated by anti-HA-conjugated Dynabeads protein G from cell lysates. Immunoprecipitated proteins were silver stained.") in the legend of Fig. S4.

5. In line 77-79 the author state "We show that non-vacuolar TORC1 has interaction with specific mRNAs and three mRNA-binding proteins among TORC1 interactome, i.e., Scd6, Ssd1, and Whi3, are phosphorylated by TORC1." Of note, Ssd1 was previously found in genetic screens to interact with TOR1 and TOR2 (PMID: 22296835 and 27708008; https://www.yeastgenome.org/locus/S000002701/interaction). Therefore, the authors should describe in their discussion the previous data about Ssd1 in relation with Tor1 and Tor2 and compare them to their findings.

➔ We appreciate the reviewer's comment. As the reviewer noted, Ssd1 has been identified as a genetic interactor of Tor1 and Tor2 in previous studies ((Alarcon et al., 1999; Costanzo et al., 2016; Reinke et al., 2004) for Tor1 and (Cardon et al., 2012; Costanzo et al., 2016) for Tor2). Following the reviewer's suggestion, we included a description about Ssd1 in the Discussion section of the revised manuscript (Lines 412-420).

6. The authors should include a reference in line 183, where they introduce liquid-liquid phase separation in mRNA granules.

➔ Following the reviewer's suggestion, we included a review paper (Brangwynne, 2013) as a reference in the revised manuscript (Line 187).

7. In lines 37 - 43 the authors claim "TORC1 regulates temporal aspects of cell growth (...). TORC2 regulates spatial aspects of cell growth by controlling the organization of the actin cytoskeleton (Loewith et al., 2002; Wullschleger et al., 2006)." This view is somewhat outdated as ample evidence has accumulated since 2006 that TORC2 is an important metabolic controller as well. See for instance the review of Roelants et al (2017) from the Thorner lab (PMID: 28872598). Please revise the statement based on the newer literature. Revise also throughout to recognize the recent literature.

➔ We appreciate the reviewer's comment. Following the reviewer's suggestion, we updated the information, included a reference (Roelants et al., 2017), and revised the statement (Lines 36-41).

8. Cite also Wippich et al (PMID: 23415227) for the statement in lines 57 - 61.

➔ Following the reviewer's suggestion, we cited (Wippich et al., 2013) paper in the revised manuscript (Line 59).

9. Please correct the typo in line 121 "throughput format and out data set is highly complementary to the existing data sets". Replace "out" with "our". The article is missing in several instances, e.g. in line 44. Please revise grammar and typos carefully throughout.

➔ We are sorry for our mistakes. We corrected grammatical errors and typos throughout the manuscript, including one in line 121. In addition, we had the manuscript copy-edited by a professional scientific editing service (American Journal Experts).

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Analysis of the TORC1 interactome reveals spatially distinct function of TORC1 in mRNP complexes

prepared by the authors

Yeonji Chang, Gyubum Lim, Won-Ki Huh

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December 16, 2020

RE: JCB Manuscript #201912060R

Dr. Won-Ki Huh
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Dear Dr. Huh:

Thank you for submitting your revised manuscript entitled "Analysis of the TORC1 interactome reveals spatially distinct function of TORC1 in mRNP complexes". The reviewers now support publication so we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below). In your final revision, you must address the final reviewer concerns with text edits and the inclusion of the indicated reviewer figures in the supplementary figures for publication. You are also encouraged if possible to include replicates for Figure S5B and the suggested coIPs with Scd6 phosphosite mutants, as well as the analysis of Kog1 behavior.

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test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

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Reviewer #1 (Comments to the Authors (Required)):

The authors have more or less addressed our previous concerns. It is unfortunate that more concrete in vivo evidence for TORC1-mediated Scd6 phosphorylation was not presented. Fig. S5B could have been supported with replicates (as the phenotype seems subtle) and, ideally, by demonstrating that the Scd6 phosphosite mutants no longer show rapamycin-induced changes in coIP.
Line 196 clarifies that 103 cells where cherry-picked to show the TORC1-P granule interaction - how many cells were screened to arrive at these 103? Is this a one in a thousand event? This should be clarified.

Reviewer #2 (Comments to the Authors (Required)):

The authors have addressed most concerns by additional experiments and/or detailed explanation. They convincingly explain the reasons why some experiments were not feasible. The findings are coherent with the model presented that a cytoplasmic RNP associated portion of TORC1 phosphorylates and regulates Scd6. In summary, the manuscript substantially improved and is of general interest.

The reviewers have some last, minor comments for clarification:
1) In line 267 the authors state "Moreover, TORC1 purified from rapamycin-treated cells could not phosphorylate Scd6 (Fig. 4C)." However P-Scd6 detection upon rapamycin is higher compared to the negative controls. Thus, "reduced phosphorylation" would be a more appropriate phrasing.

2) The finding presented in Figure 3F on RNA-mediated interaction between Tor1 and p-body components is important. To further strengthen the statement on TORC1-p-body association, it would be valuable to show if Kog1 behaves in the same manner.

3) To further increase the impact of the manuscript, we propose to include some of the data presented in the "Response to Reviewers", in particular those in R14 and R15. These data could be shown in the supplemental figures.

Response to the reviewers' comments

Reviewer #1 (Comments to the Authors (Required)):

The authors have more or less addressed our previous concerns. It is unfortunate that more concrete in vivo evidence for TORC1-mediated Scd6 phosphorylation was not presented. Fig. S5B could have been supported with replicates (as the phenotype seems subtle) and, ideally, by demonstrating that the Scd6 phosphosite mutants no longer show rapamycin-induced changes in coIP.

➔ We thank the reviewer for his/her thoughtful and perceptive comments. As the reviewer suggested, we repeated experiments for Fig. S5B and showed the mean ± SD of duplicate experiments.

Line 196 clarifies that 103 cells where cherry-picked to show the TORC1-P granule interaction - how many cells were screened to arrive at these 103? Is this a one in a thousand event? This should be clarified.

➔ We examined 245 cells after 30 min of glucose starvation and found 103 cells that exhibited the colocalization of Tor1 foci and Dcp2. We included this this information in the legend of Fig. 3B in the revised manuscript (line 779-780).

Reviewer #2 (Comments to the Authors (Required)):

The authors have addressed most concerns by additional experiments and/or detailed explanation. They convincingly explain the reasons why some experiments were not feasible. The findings are coherent with the model presented that a cytoplasmic RNP associated portion of TORC1 phosphorylates and regulates Scd6. In summary, the manuscript substantially improved and is of general interest.

➔ We appreciate the reviewer's thoughtful and considerate comments.

The reviewers have some last, minor comments for clarification:
1) In line 267 the authors state "Moreover, TORC1 purified from rapamycin-treated cells could not phosphorylate Scd6 (Fig. 4C)." However, P-Scd6 detection upon rapamycin is higher compared to the negative controls. Thus, "reduced phosphorylation" would be a more appropriate phrasing.

➔ As suggested by the reviewer, we rephrased the sentence in the revised manuscript (line 273-274).

2) The finding presented in Figure 3F on RNA-mediated interaction between Tor1 and p-body components is important. To further strengthen the statement on TORC1-p-body association, it would be valuable to show if Kog1 behaves in the same manner.

➔ We confirmed that Kog1 also behaves in the same manner as Tor1. However, because the data quality was not good enough for publication, we decided not to include the data

in the manuscript. Instead, we included the data for the Interaction between Kog1 and P-body components under glucose starvation in Fig. S3C.

3) To further increase the impact of the manuscript, we propose to include some of the data presented in the "Response to Reviewers", in particular those in R14 and R15. These data could be shown in the supplemental figures.

➔ As suggested by the reviewer, we included Fig. R15 in the revised manuscript as Fig. S5G. By the way, Fig. R14 is a model we speculate for the regulation of Scd6 by TORC1. Because several issues in the figure should be clarified in more detail, we are reluctant to show the figure in this manuscript. In addition, there is a restriction on the number of supplementary figures. Thus, we decided not to include Fig. R14 in the manuscript. At any rate, we thank the reviewer for the suggestion.