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TORC1 and TORC2 converge to regulate the SAGA co-activator in response to nutrient availability

Thomas Laboucarié, Dylane Detilleux, Ricard A. Rodriguez-Mias, Céline Faux, Yves Romeo, Mirita Franz-Wachtel, Karsten Krug, Boris Maček, Judit Villén, Janni Petersen, and Dominique Helmlinger

Corresponding author: Dominique Helmlinger, CNRS

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

1st Editorial Decision 04 August 2017

Thank you for the transfer of your research manuscript to EMBO reports.

As my colleague from The EMBO Journal has already outlined, we are potentially interested in publishing a further revised version of your work in EMBO reports. Please re-write the manuscript to better discuss the limitations of the data as outlined by referee #1 and #3 and adapt the model to the data. We will subsequently consult once more with referee #1 on such a revised version.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

1st Revision - authors' response 05 August 2017

Responses to the referees' comments:

Referee #1:

In this resubmission, the authors provided additional evidence to support that TORC2 regulates the phosphorylation of the SAGA component Taf12 and this phosphorylation is involved in the response to starvation and sexual differentiation. Since the Taf12 ala mutants increase the expression of

genes involved in differentiation whereas the phosphomimetic diminishes their expression and cell differentiation, they propose a model wherein TORC2 and Taf12 phosphorylation could be controlling the amplitude of the response to starvation. The additional data are somewhat supportive of their model. One thing that remains confusing is the proposal that TORC2 both promotes and inhibits sexual differentiation. One thing that should perhaps be considered in order to reconcile the puzzling observations is that the phosphorylated Taf12 prevents its nuclear localization and thus negatively modulates differentiation genes. Indeed, it appears that Taf12 expression is enhanced during prolonged starvation (Figure 3I) while proportion of phosphorylated Taf12 decreases. In line with this, it is likely that this increased expression (and possibly nuclear localization) is modulated by mTORC2. This would then be in favor of TORC2 and dephosphorylated Taf12 as a positive regulator of differentiation during starvation.

The phosphorylation data are interesting and together with the additional data on Figure 7 reveal new aspects of TORC2 regulation of differentiation. It is more the presentation of their model and interpretation of the data that makes the study quite confusing, and therefore needs improvement. The discussion on page 21 on opposing activities of Fkh2 and Taf12 is highly speculative. The data are supportive of TORC2 and Taf12 as positive regulators of differentiation during starvation. The evidence that TORC2/Gad8 mediate the phosphorylation of Taf12 leading to its inhibition of differentiation is not convincing. What is convincing is that the lack of Taf12 phosphorylation enhances differentiation. At the very least the authors should clarify their model on Figure 7 to accurately illustrate their point on TORC2 modulating the amplitude of response to starvation via Taf12 phosphorylation. Such a model would reveal that in fact TORC2 is positively regulating Taf12 and that Taf12 (unphosphorylated) is a positive regulator of differentiation.

Response:

We thank the referee for constructively extending our model to reconcile the puzzling observations that TORC2 both promote and inhibit sexual differentiation. We have included it in a novel Figure 7F and discussed it on Page 22 of the Discussion (1st paragraph). It is indeed conceivable that TORC2 inhibitory function is restricted to the early time points of nutrient starvation (Left panel in Figure 7F). Then, at later time points, TORC2 promotes differentiation through the mechanism proposed by the referee (right panel in Figure 7F). Again, we would like to thank the referee for clearly improving our manuscript and have explicitly acknowledged his/her contribution to this model in the Acknowledgments section (Page 30). We respectfully argue that an additional possibility is that TORC2-Gad8AKT controls both Taf12 and Fkh2, in what would appear as an incoherent feed-forward loop. As mentioned above, this hypothesis is not mutually exclusive with the referee's model. We proposed it based on the following evidence:

- Our data show that Taf12 phosphorylation is induced early upon starvation, positively correlates with TORC2 activity, but not any other nutrient-sensing pathway, and negatively correlates with sexual differentiation.

- The Fkh2 transcription factor induces *ste11+* expression and sexual differentiation upon starvation (for example, see Figure 1 from Shimada M. *et al*., The EMBO Journal (2008) 27, 132–142). Similar to many mammalian forkhead transcription factors, Fkh2 is a robust substrate for the Gad8AKT kinase *in vitro* (first reported in Figure 4 from Ikeda K. *et al*., Cell Cycle 92008) 7:3, 1-7). Last, phosphorylation of Fkh2 on Ser321 by Gad8AKT has recently been shown to be required for sexual differentiation (see Figure 5G from Martin R *et al*., Current Biology (2017) 27, 1–14).

- The idea that TORC2 can also inhibit sexual differentiation is now shown here for the first time and was already demonstrated in Hálová L. et al., J. Cell Biol. (2013), 203, 4, 595–604. Importantly, our work now provides a mechanism for how this inhibitory function is achieved.

We have amended the Discussion on Pages 20-21 to clarify the rationale for this hypothesis, removing our sentence about "*TORC2-activated Gad8AKT phosphorylates Taf12 to inhibit differentiation.*"

Referee #3:

A major conclusion of the study is that the Gad8 kinase downstream of TOR complex 2 (TORC2) phosphorylates Taf12 to negatively control expression of the key genes in sexual differentiation of S. pombe. In the last revision, the authors showed that alanine substitutions of the Gad8 phosphorylation sites in Taf12 (taf12-5A) did not affect the expression of ste11+ and mei2+ after 4 hours of starvation (Supplemental Figure 14 of the previous manuscript). The current manuscript presents a new data indicating that those key genes in sexual differentiation are derepressed in the taf12-5A mutant around 2 hours after starvation (Figure 7A&B). Because the Taf12 phosphorylation is high around 2 hours after starvation but decreases by 4 hours (Figure 3I), the authors propose that the TORC2-Gad8 mediated phosphorylation of Taf12 plays an important role in preventing cells from committing to sexual differentiation early upon nutrient starvation (~ 2 hours after starvation).

However, other important data is included in the current manuscript, showing that, unlike taf12-5A, the ste11+ and mei2+ expression is not derepressed in the gad8Δ mutant at 2 hours after starvation (Supplemental Figure 9). This observation indicates that the Gad8-mediated phosphorylation of Taf12 is not required for repression of the sexual differentiation genes, negating the authors' major conclusion of the manuscript. It should be noted that alanine is not a perfect mimic of unphosphorylated serine/threonine. Therefore, alanine substitution of phosphorylation sites needs to be interpreted with caution, in combination with careful analysis of the phenotype caused by inactivation of the kinase responsible for the phosphorylation. It is possible that the alanine mutations in Taf12-5A causes a conformational change within SAGA and/or TFIID, affecting the transcription of the target genes.

Response:

First, Gad8AKT has likely other substrates than Taf12, including at least Fkh2. Therefore, a direct comparison of the phenotype of cells in which the gene encoding a kinase is deleted with the phenotype of cells in which one, and only one, of its substrate is mutated can be problematic, particularly considering that the TORC2-Gad8AKT pathway both inhibits and promotes sexual differentiation (this paper and Hálová L. et al., J. Cell Biol. (2013), 203, 4, 595–604).

Second, we respectfully disagree with the following statement from the referee: “This observation indicates that the Gad8-mediated phosphorylation of Taf12 is not required for repression of the sexual differentiation genes, negating the authors' major conclusion of the manuscript.” The phenotypic difference pointed by the referee suggests either that Gad8AKT has other substrates as mentioned above, which we know is true, or that TORC2 mediates the phosphorylation of Taf12 and inhibition of sexual differentiation through another kinase than Gad8AKT. We now discuss this point explicitly (Page 22, end of 2nd paragraph).

Third, as discussed in the 2 previous rounds of the revision, it is formally possible that substituting Ser/Thr into Ala causes a phenotype unrelated to phosphorylation, but, to the best of our knowledge, this remains the standard method to assess the effect of phosphorylation in vivo. Nevertheless, we acknowledge this possibility (Page 18, last paragraph of the Results section), but we have no evidence that these mutations significantly alter Taf12 function. The possibility that these mutations “cause a conformational change with SAGA and/or TFIID” is, word for word, what we propose as a mechanism for how phosphorylation controls SAGA/TFIID activities.

The authors also introduced in this latest manuscript an activated allele of tor1, tor1-T1972A, as additional evidence that enhanced TORC2-Gad8 signaling can inhibit sexual differentiation (Figure 7E). However, the experiment examined the effect of the tor1-T1972A mutation after 48 hours of starvation and is irrelevant to the discussion on the specific role of TORC2-Gad8 signaling early upon starvation. In addition, the compromised sexual differentiation in the tor1-T1972A mutant has been attributed to its defect in G1 cell cycle arrest (Halova et al., 2013) and it remains to be determined if the mutation affects the transcription of ste11+ and mei2+.

Response:

First, in our experimental conditions, we find it impossible to detect a significant proportion (>1%) of differentiating cells at 1 or 2 hours of starvation. Additionally, we generated the tor1-T1972A taf12-5A mutants in heterothallic h- and h+ backgrounds to avoid having to entirely re-construct these mutant alleles from scratch in a homothallic h90 background, which is difficult to use to cross different strains between them. As a result, we had to perform the differentiation assay on SPAS plate, as detailed in the Materials and Methods (Page 25). In these experimental conditions, it is impossible to measure the exact time spent by cells in starvation, as opposed to inoculating homothallic h90 cells in starved liquid medium from exponentially growing cultures. Therefore, our annotation in Figure 7E (Starved (48h)) is not accurate and was removed from the new version of Figure 7. We apologize for the confusion and clarify this point in the legend to Figure 7. Importantly, Figure 7D clearly shows that Taf12 phosphorylation increases in hyperactive TORC2 mutants after 1 hour of starvation.

Second, the majority of sterile fission yeast mutants also have G1 cell cycle arrest defects. These processes are intricately linked and the master regulator of sexual differentiation, Ste11, is required for G1 exit (Kjaerulff S. et al., Mol. Cell. Biol. (2005) 25:2045-2059).

Minor point:

p.20, lns 15-17. "It is unlikely that PP2A controls Taf12 phosphorylation indirectly, by inhibiting Gad8, because PP2A directly de-phosphorylates Taf12..." Is this statement based on the in-vitro dephosphorylation experiment shown in Figure 4E? As most phosphatases do not have very strict substrate specificity, this type of in-vitro experiments does not serve as evidence for direct dephosphorylation in vivo. The experiment only shows that PP2A is capable of dephosphorylating Taf12 at least in vitro. Thus, the overstatement above needs to be rectified.

Response:

This statement is based on the in vitro de-phosphorylation experiment shown in Figure 4E and on the in vivo evidence that Taf12 phosphorylation depends on PP2A-Pab1 but further increases upon starvation of pab1D mutants (Figure 4D). The latter observation suggests that a kinase still responds to starvation in the absence of PP2A-Pab1 to phosphorylate Taf12. We were unable to generate a pab1D gad8D double mutant because it is not viable. To control for specificity of our phosphatase assay, we used Pab1 purifications to pull down only a fraction of PP2A, and microcystin to inhibit its activity. In vitro experiments are indeed not evidence for in vivo regulation, but, then, in vivo observations do not provide evidence for a direct regulation. The sentence has nevertheless been rectified accordingly (Page 20, 2nd paragraph) to “Our work reveals that an additional substrate of the TORC1-PP2AB55 pathway contributes to this control this process.”

2nd Editorial Decision 28 August 2017

Thank you for the submission of your revised manuscript to EMBO reports. As you will see, former referee #1 has now reviewed your revised manuscript and supports its publication in EMBO reports.

Browsing through the manuscript myself I noticed a few things that we need from the editorial side, before we can proceed with the official acceptance of your study. Please:

- Shorten the title to 100 characters incl. spaces and the abstract to 175 words.
- Remove the text on "Expanded view files" on page 29.
- Provide a paragraph on Author contributions and a Conflict of interest statement
- Update the references to match the numbered style of EMBO reports.
- Review the legend for Fig 4: it states "A-H" while the figure only contains panels "A-G"
- Rename the Appendix and add a table of content with page numbers (see our Guide to Authors).
- Add a callout for Table S2. It is currently never mentioned in the text.
- Provide the revised manuscript as a .doc file and upload the figure files in high resolution.
- Provide a completed author checklist.

I look forward to seeing a final version of your manuscript as soon as possible.

REFEREE REPORTS

Referee #1:

The authors have addressed the previous comments from this reviewer and have clarified their model.

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Corresponding Author Name: Dominique HELMLINGER
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2017-44942V2

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This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
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- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
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- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

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Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For all experiments, the plausibility of the H0 hypothesis was estimated to be at a 1-to-1 odd ratio, meaning that we did not have any pre-conceived guess of the outcome of an experiment. Sample size was chosen based on previous experimental knowledge, which showed that n >= 4 was sufficient to detect statistically significant differences if the effect is >= 2-fold.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	All samples analyzed were included in all analyses, without excluding any particular results, except if the outcome was technically impossible to interpret.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Overall, all experiments were performed using strain numbers, but not genotype annotations, to label each sample, reducing a possible bias when handling the samples.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes, see details in the corresponding section of Materials & Methods, page 34. In addition, each figure legend details which test was used, which alpha treshold was decided a priori, and the number of biological replicates. Finally, most data are presented as the mean +/- standard error (SE), overlaid with individual data points.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We performed parametic tests (ANOVA and t-tests) using Graphpad Prism 5. Therefore, we indeed assumed that all our data meet the following assumptions: normal distribution, equal variances, and independence of biological replicates. Normality is typically observed for continuous, relative measurements of expression (RT-qPCR) or intensity levels (Western blotting). Visual examination of our data suggest that samples have similar variances. However, we did not run dedicated statistical tests, such as Shapiro-Wilk test to test for normality or the Bartlett's test for variance equality.
Is there an estimate of variation within each group of data?	Variation was estimated by computing and showing the standard error of the mean (SE), as well as visually, by overlaying individual data points whenever possible.

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Is the variance similar between the groups that are being statistically compared?	We assume that the variance is similar between the groups that were statistically compared, based on previous experiemental knowledge and visaul inspection of the data points. However, this was not tested by a dedicated statistical test (such as the Bartlett's test).

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Proper citations, catalog number and, when available, clone number are included in the corresponding sections of Materials & Methods, page 29.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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D- Animal Models

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18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
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