EMBO reports

Branched-chain amino acid synthesis is coupled to TOR activation early in the cell cycle in yeast

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DOI: 10.15252/embr.202357372

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Review Timeline:

Transfer from Review Commons:	21st Apr 23
Editorial Decision:	12th May 23
Revision Received:	16th Jun 23
Editorial Decision:	30th Jun 23
Revision Received:	5th Jul 23
Accepted:	7th Jul 23

Editor: Ioannis Papaioannou

Review COMMONS

Transaction Report: This manuscript was transferred to EMBO reports following peer review at Review Commons.

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Review #1

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary

In this manuscript, Blank et al. propose a link between cell-cycle dependent changes in metabolic flux and corresponding changes in TORC1 activity in yeast cells. Based on their findings, the authors propose that Bat1-dependent leucine synthesis from glucose increases as cells progress through G1 and that this activates TORC1 to drive cell cycle progression. Although the existence of cell-cycle dependent synthesis of leucine is a novel and exciting finding, several aspects of the proposed model are not sufficiently supported by experimental evidence, in particular the fact that the increase in Leu synthesis is causing the increase in TORC1 activity in late G1.

Major comments:

1. To show that the increase in Leu biosynthesis in S-phase is activating TOR, one would ideally want to blunt this increase in biosynthesis and assay TORC1 activity. Admittedly, this is difficult. So, instead, the authors study bat1- cells which have strongly impaired synthesis of BCAA including Leucine. The relevance of these bat1- cells to the proposed cell-cycle dependent model, however, is questionable for two reasons: 1) Although the authors state that "exogenous supplementation of BCAAs in all combinations suppressed the growth defect of bat1- cells, especially when valine was present", the spot assays in Figure 3 show visible rescues only when valine is present either alone or in combination, while supplementation of leucine or isoleucine does not seem to have any effect. Hence it appears that the bat1- phenotype is mainly due to limiting valine levels, not leucine levels. 2) The relevance of these results for understanding TORC1 regulation are questionable, since valine does not typically activate TORC1. Does additionn of Leu to bat1- cells increase TORC1 activity ?
2. TORC1 activity is known to depend on steady-state leucine concentrations in the cell rather than on leucine flux. Although the authors observe that the synthesis rate of leucine increases during G1 progression, this does not necessarily translate innto increased leucine concentrations in the cell. To support the claim that the increase in TORC1 activity during G1 progression depends on leucine, the authors would need to show that, not only leucine synthesis, but also overall leucine levels in the cell increase during G1 progression.
3. To test whether the increase in Leu biosynthesis in S-phase activates TORC1, a few different approaches could be tested: 1) Since leucine activates TORC1 through the Gtr proteins, the authors could test whether rendering TORC1 resistant to low leucine through expression of constitutively active Gtrs abolishes the cell-cycle dependence in TORC1 activity. 2) Leu could be added to the medium of wildtype cells in G1 to the amount necessary to cause an increase in intracellular Leu levels similar to those seen in S-phase to test whether this increases TORC1 activity.
4. In Fig 2B one sees that Leu biosynthesis peaks at 150min and then drops again. The

p-RpS6 blot in Fig. 5D, however, only goes up to 140 min and shows that TORC1 activity increases up to 140 min, but it doesn't show timepoints beyond 150 min when Leu biosynthesis drops again, and hence one would expect TORC1 activity to drop. If TORC1 activity were to drop from 150min onwards, this would strengthen the correlation between Leu biosynthesis and TORC1 activity.

Minor concerns:

1. In Figure EV3, the authors should highlight some of the metabolites that are significantly changed, in particular the BCAA. The figure is not very informative as currently presented.
2. Fig 2 - are "expressed ratios" the best term for metabolite levels? Unlike genes, where such heat maps are often used, the metabolites are not 'expressed'. How about 'relative metabolite level' instead?
3. Page 8: "We also measured the MID values from the media of the same cultures used to prepare the cell extracts." Where are these data? We don't see them in File S2?
4. Fig 4B - the x-axis labeling is missing for the bat1- cells
5. Although the authors state repeatedly that they show "for the first time in any system" that TORC1 activity is dynamic in the cell cycle, similar observations have already been made before, for instance showing high mTORC1 activity in the G1/S transition in the Drosophila wing disc or low mTORC1 activity during mitosis in mammalian cells (see PMIDs 28829944, 28829945, and 31733992). The text should be amended accordingly.
6. There are two entries for valine in File S1/Sheet8. Why?

2. Significance:

Significance (Required)

Despite the well-known effects of pharmacological or genetic manipulations of TORC1/mTORC1 on cell cycle progression, whether and how mTORC1 activity itself is physiologically coupled to cell cycle progression is still an insufficiently studied aspect. Hence this study provides an interesting link between cell-cycle dependent regulation of amino acid biosynthesis and TORC1 regulation. Importantly, the results of this study rely on centrifugal elutriation to obtain cell cycle synchronization, thus ruling out potential metabolic artifacts due to pharmacological methods. The observed changes in metabolic flux are therefore likely genuine and represent the major strength of the study. The major limitation is the lack of strong evidence supporting the notion that the increase in Leu biosynthesis at late G1 or S-phase is causing the increase in TORC1 activity.

The major advance is conceptual - that amino acid biosynthesis rates are cell-cycle dependent.

These results will be of interest to a broad audience of people studying the cell cycle, cell growth, TORC1 activity, cell metabolism and cancer.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

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No

Review #2

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

This paper provides evidence that branched chain amino acid (BCAA) in the G1 phase of the cell cycle, fueled by pyruvate generated by glucose catabolism activates cell growth and allows cells to reach the critical size required for entry into S phase by activation of TORC1 signaling. Previous work had indicated that Leucine supplementation of a bat1 bat2 mutant, lacking both enzymes that catalyze BCAA from the alpha-keto acid precursors and starved on minimal medium, led to TORC1 activation. This work is significant in suggesting that BCAA synthesis from glucose is responsible for a cyclic activation of TORC1 necessary for a normal rate of cell growth in the G1 phase of the cell cycle.

The study employs metabolic flux analysis of metabolites derived from glucose following a pulse-chase with different isotopes of glucose in synchronized early G1 cells (obtained by elutriation) throughout one cell cycle. They claim that the only compelling changes in metabolites observed as the cell cycle proceeds was a decline in pyruvate

containing only one heavy 13C carbon atom and a corresponding increase in Leu (M6) with 6 heavy carbon atoms, which is interpreted to indicate Leu synthesis from pyruvate that begins in early G1 and peaks at mitosis. They show that a bat1 mutant exhibits a slow-growth phenotype that can be mitigated only by valine (although they infer similar effects for Leu and Ile that I find unconvincing) and they observed reductions in all three BCAAs in different experiments that measure steady amino acid levels in different ways (although the results are compelling only for Val). They go on to show evidence that the bat1 mutation reduces birth and mean cell size and leads to an increased proportion of G1 cells in asynchronous cultures, and they claim that bat1 cells take much longer than WT to achieve the same size found when a synchronized WT culture reaches 50% budding (although they don't show the data for this last point.) Interestingly, they find that deleting BAT1 suppresses sensitivity to the TORC1 inhibitor rapamycin (Rap), consistent with the idea that the bat1 mutation impairs TORC1 activity in the same manner as Rap and that BCAA are required to activate TORC1 in WT cells to the level that can be impaired by Rap, as summarized in the model in Fig. 5F. Consistent with this, they present evidence that the bat1 mutation reduces TORC1 signaling as judged by diminished Rps6 phosphorylation (although it was not shown that this effect could be reversed by Val addition). They also show that TORC1 signaling/Rps6-P increases as the cell cycle progresses using elutriated early G1 cells, suggesting that TORC1 activity is periodic in the cell cycle (although they don't establish this periodicity through a second cell cycle).

General critique:

The conclusion that BCAA synthesis from glucose is responsible for a cyclic activation of TORC1 necessary for a normal rate of cell growth in the G1 phase of the cell cycle is potentially of considerable significance. There are however a number of puzzling aspects of the data that seem to weaken this conclusion. As described in greater detail below, it is difficult to explain why only Leu is synthesized from glucose during the cell cycle, and why only Val shows a marked reduction in the bat1 mutant that appears to be responsible for the slow-growth phenotype. In addition, there are important controls lacking of showing that a Val supplement can suppress the G1 delay and reduction in TORC1 signaling in the bat1 mutant. In addition, the evidence that TORC1 activity is periodic in the cell cycle is lacking and it needs to be shown that Rps6-P levels are periodic through at least a second cell cycle.

Major comments:

- Why don't they observe synthesis of Ile and particularly Val in the metabolic flux experiment of Fig. 1, especially considering that only Val appears to be critically required for normal cell growth in the bat1 mutant based on the results in Fig. 3B?
- The data in Fig. 3B do not show a convincing increase in growth of the bat1 mutant with addition of Leu and Ile; and the stimulation by Val alone seems identical to that seen with Val in combination with Leu and Ile. Thus, it appears that the slow-growth of the bat1 mutant results only from reduced Val levels, not all 3 BCAAs, which is at odds with their interpretation of the data.
- they claim to see reductions in all three BCAAs in the bat1 mutant; however, no significant reduction was found for Leu in Fig. EV2, and only Val was altered by the 1.5-fold cut-off imposed on the MS metabolomics data in Fig. EV3 (which could be

appreciated only by an in-depth examination of the supplementary data in File S1-the Val, Leu, and Ile dots should be labeled in Fig. EV3). In addition, the reductions in Ala and Gly showin in Fig. EV2 were not found in the MS analysis of Fig. EV3. It needs to be acknowledged that the metabolomics data show a marked reduction in the bat1 mutant only for Valine with little or no change in Leucine levels. This result is difficult to explain with the simple models shown in Fig. 3A and 5F, which requires additional comment. The authors should acknowledge the much greater effect of the bat1 mutation on Val levels versus Leu and Ile, revealed both by measuring the levels of BCAAs in the mutant and comparing the BCAAs for rescuing the slow-growth of the mutant, and explain how this can be reconciled with the results in Fig. 2 where only Leu and not Val or Ile synthesis was detected.
- They need to add the data indicating that the bat1 mutant requires longer than WT cells to reach the ~35 fL volume at which 50% of WT cells are budded.
- It seems important to show that Val supplementation can suppress the overabundance of G1 cells in bat1 mutant cells shown in Fig. 4C; and can restore sensitivity to Rap and Rps6-P accumulation in bat1 mutant cells (in Fig.s 5A & B).
- It seems important to show that Rps6-P will decline in M phase and increase during a second cell cycle to establish that TORC1 activity actually fluctuates in the cell cycle instead of just by reduced by the manipulations involved in collecting young G1 cells by elutriation.

Referees cross-commenting

Ref. #1's major comment 1 echoes my request for clarification about whether Leu, and not just Val, is limiting growth in the bat1 mutant, and also the need to determine which BCAA supplement to bat1 cells will restore TORC1 activity (which was also requested by Ref. #3). I agree with this reviewer's request to provide evidence that Leu levels actually increase during G1 progression (comment #2). I also think the suggested experiments in Comment #3 are reasonable for their potential to provide stronger evidence that Leu production in the G1 phase of wild-type cells activates TORC1, as currently the argument is based on the finding of low TORC1 activation in bat1 cells (that seem to be limiting for Val vs. Leu). Comment #4 echoes similar requests made by both me and Ref. #3.
Ref. #3's major comments 1 and 3 mirror two of my major comments. I wasn't convinced of the need to monitor Sch9 versus Rps6 phosphorylation as a read-out of TORC1 activity-does being a direct substrate truly matter? Regarding comment 5, I wasn't convinced of the need to include Rap-sensitive or -resistant control strains for the analysis in Fig. 5A. And regarding comment 4, while it would be interesting to examine if TORC1 regulates BCAA synthesis during cell cycle progression, this seems to be outside the scope of a demonstration that BCAA synthesis stimulates TORC1.

Thus, it seems we all agree on certain experiments that need to be carried out, and Ref. #1 has rightly proposed a few others with the potential to strengthen the evidence that Leu production during G1 phase mediates cyclic activation of TORC1

2. Significance:

Significance (Required)

General Assessment:

Strengths: Evidence for BCAA biosynthesis from glucose in the G1 phase of the cell cycle, and evidence obtained from analyzing the bat1 mutant that BCAA synthesis underlies activation of TORC1 early in the cell cycle in a manner required to achieve the critical cell size necessary for G1 to S transition.

Weaknesses: Lack of evidence for Val biosynthesis in G1 despite evidence that Val limitation is more crucial than Leu limitation in the bat1 mutant; lack of confirmation that Val limitation underlies the delayed G1-S transition and reduced TORC1 signaling in the bat1 mutant; and lack of compelling evidence that TORC1 activity is periodic in WT cells.

Advance: This would be the first evidence that TORC1 activity varies through the cell cycle in a manner controlled by synthesis of BCAAs

Audience: This advance would be of great interest to a wide range of workers studying how the cell cycle is regulated and the role of TORC1 in controlling cell growth and division in normal cells and in human disease.

My expertise: Mechanisms of metabolic regulation of gene expression at the transcriptional and translational levels in budding yeast

3. How much time do you estimate the authors will need to complete the suggested revisions:

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Review #3

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

In this manuscript, Blank and colleagues measure the synthesis of various metabolites from glucose during cell cycle progression and observe an increased synthesis of branched-chain amino acids (BCAA) from the early G1 to late G1 phase. Interestingly, they also found a gradual increase in TORC1 activity from the early G1 to the S phase which is proposed to be dependent on BCAA synthesis.

Major comments:

1. The authors show that TORC1 activity increases from the early G1 to the S phase. TORC1 activity is sensitive to short-term starvations caused during changing media or centrifugations. Hence, the concern arises regarding the increased pattern of TORC1 activity during the cell cycle. Is it really a biological phenomenon or a cellular adaptation to experimental conditions? Can authors provide more support for this observation? Can authors monitor the cell cycle for the two cell cycles to confirm that TORC1 activity shows a wavy pattern?
2. The authors use Rps6 phosphorylation as a read-out of TORC1 activity, which is not a direct substrate of TORC1. Analysis of the direct substrates of TORC1, such as phosphorylation of Sch9 will solidify the author's claim.
3. Authors show that Bat1 lacking strain have reduced TORC1 activity. Can authors restimulate these cells with Leucin, Valine, and Isoleucine individually or in combination to identify the critical amino acid for the TORC1 activity?
4. The authors claim that increased BCAA synthesis is necessary for TORC1 activation. Since TORC1 is shown to be upstream of amino acid biosynthesis pathways, it will be interesting to check if TORC1 per se regulates BCAA synthesis during cell cycle progression. The authors could inhibit TORC1 by rapamycin treatment and monitor if the BCAA synthesis still shows cell cycle-dependent modulation.
5. In Figure 5A, the use of any rapamycin-sensitive and rapamycin-resistant strains as controls will strengthen their claim of TORC1 inhibition being epistatic to Bat1 deletion, since the rapamycin in minimal media might be less effective.

Minor comments:

1. The data of metabolic labeling, especially various species M1, M2, M3, etc., of an individual metabolite is difficult to understand for the general readers. Hence, a schematic explaining various species might be helpful.
2. Please describe the elutriation approach in more detail with media conditions and buffer conditions to understand the overall experimental setup.

2. Significance:

Significance (Required)

Overall, this study presents an interesting observation to the researchers working in TORC1 and cell cycle regulation.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

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Full Revision

Manuscript number: RC-2023-01825

Corresponding author(s): Michael Polymenis and Karsten Hiller

1. General Statements

We thank all reviewers for their comments and suggestions. The revised manuscript included new experiments they suggested and extensive text edits. Our point-by-point response is shown in bold.

2. Point-by-point description of the revisions

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Reviewer #1 (Evidence, reproducibility and clarity (Required)):

Summary
In this manuscript, Blank et al. propose a link between cell-cycle dependent changes in metabolic flux and corresponding changes in TORC1 activity in yeast cells. Based on their findings, the authors propose that Bat1-dependent leucine synthesis from glucose increases as cells progress through G1 and that this activates TORC1 to drive cell cycle progression. Although the existence of cell-cycle dependent synthesis of leucine is a novel and exciting finding, several aspects of the proposed model are not sufficiently supported by experimental evidence, in particular the fact that the increase in Leu synthesis is causing the increase in TORC1 activity in late G1.

Major comments:
1. To show that the increase in Leu biosynthesis in S-phase is activating TOR, one would ideally want to blunt this increase in biosynthesis and assay TORC1 activity. Admittedly, this is difficult. So, instead, the authors study bat1- cells which have strongly impaired synthesis of BCAA including Leucine. The relevance of these bat1- cells to the proposed cell-cycle dependent model, however, is questionable for two reasons: 1) Although the authors state that "exogenous supplementation of BCAAs in all combinations suppressed the growth defect of bat1- cells, especially when valine was present", the spot assays in Figure 3 show visible rescues only

when valine is present either alone or in combination, while supplementation of leucine or isoleucine does not seem to have any effect. Hence it appears that the bat1- phenotype is mainly due to limiting valine levels, not leucine levels. 2) The relevance of these results for understanding TORC1 regulation are questionable, since valine does not typically activate TORC1. Does addition of Leu to bat1- cells increase TORC1 activity ?

RESPONSE: The reviewer's comments were very valuable. We performed the suggested experiments (adding not only Leu but also Ile and Val) to bat1 cells and measuring phosphorylation of Rps6 (see new Figure 4D) and the DNA content of those cells (see new Figure 3C). We found that Leu weakly promotes cell cycle progression, compared to the addition of Val, which also leads to pronounced activation of TORC1 (>10-fold activation; see Figure 4D). We discuss these findings in the revised text.

We also note, as published by others and now discussed in the text, that in WT cells, exogenous addition of Leu (or any other BCAA) does not lead to sustained activation of TORC1 (see new Figure 4D). This is not surprising. As reported by the Hall lab (see PMID: 25063813, which we now cite), the Gtr-dependent activation of TORC1 by BCAAs mentioned by the reviewer is very transient. Hence, our new data, showing sustained TORC1 activation and cell cycle effects upon Val addition in bat1 cells, is exciting. They argue that bat1 cells serve as a highly sensitized background of low TORC1 activity, enabling the display of effects that are difficult to measure in WT cells.

2. TORC1 activity is known to depend on steady-state leucine concentrations in the cell rather than on leucine flux. Although the authors observe that the synthesis rate of leucine increases during G1 progression, this does not necessarily translate into increased leucine concentrations in the cell. To support the claim that the increase in TORC1 activity during G1 progression depends on leucine, the authors would need to show that, not only leucine synthesis, but also overall leucine levels in the cell increase during G1 progression.

RESPONSE: We did this experiment and now report the data (see new Figure EV2), using the Edman degradation-based assay. We found that changes in the steady-state levels of BCAAs had a similar pattern, and those changes were most significant for valine (rising 30-40% from late G1 to G2/M). Nonetheless, we note also that the kinetics of amino acid synthesis measured by our isotope tracing experiment need not match the steady-state levels of amino acids. Steady-state levels are affected by a multitude of parameters, only one of which is the rate of synthesis, as we now discuss in detail in the manuscript.

3. To test whether the increase in Leu biosynthesis in S-phase activates TORC1, a few different approaches could be tested: 1) Since leucine activates TORC1 through the Gtr proteins, the authors could test whether rendering TORC1 resistant to low leucine through expression of constitutively active Gtrs abolishes the cell-cycle dependence in TORC1 activity. 2) Leu could be added to the medium of wildtype cells in G1 to the amount necessary to cause an increase in

intracellular Leu levels similar to those seen in S-phase to test whether this increases TORC1 activity.

RESPONSE: We did the suggested experiments, which are now shown in the new Figure 5. Leucine and valine accelerated the rise in TORC1 activity in G1. However, there were no noticeable downstream consequences in the kinetics of cell cycle progression. As we discuss in the text:
"A small acceleration of the rise in the levels of phosphorylated Rps6 was evident in both the leucine- and valine supplemented cells (Figure 5A,B). Nonetheless, there were no noticeable downstream consequences in the kinetics of cell cycle progression, in either the rate the cells increased in size or their critical size (Figure 5A; see values above the corresponding blots), consistent with the notion that TORC1 activity already is at a maximal level in these conditions…"

4. In Fig 2B one sees that Leu biosynthesis peaks at 150min and then drops again. The p-RpS6 blot in Fig. 5D, however, only goes up to 140 min and shows that TORC1 activity increases up to 140 min, but it doesn't show timepoints beyond 150 min when Leu biosynthesis drops again, and hence one would expect TORC1 activity to drop. If TORC1 activity were to drop from 150min onwards, this would strengthen the correlation between Leu biosynthesis and TORC1 activity.

RESPONSE: The reason for the drop in Figure 2 is trivial and does not affect the interpretation. As seen in Figure 1 (the experiment from which the data in Figure 2 are shown), by 180 min, the cells were entering a new cell cycle, evidenced by a reduction in cell size (Figure 1B) and in the fraction of budded cells (Figure 2B). At that point, there is a mix of mothers and daughters with very poor synchrony, making it impossible to conclude much about the drop in Leu synthesis (i.e., does it arise from the lack of new synthesis in mothers, daughters, or both?). In the experiment in Figure 5, the reviewer mentions (now those figures have moved to File S8 because we added more experiments in the figure) the experiment terminated when peak budding was reached, which was 140 min, within one cell cycle. Lastly, it is important to stress that every elutriation experiment is different. While the times are close, comparing various experiments on a time basis alone is inaccurate. Instead, the metric used in the field to compare different experiments is usually cell size, which we use in all other Figures except Figure 1 because, in that case, the experiment was a time-based, pulse-chase one.

Minor concerns:

1. In Figure EV4, the authors should highlight some of the metabolites that are significantly changed, in particular the BCAA. The figure is not very informative as currently presented.

RESPONSE: We have now labeled the BCAAs, and a few more metabolites as suggested (note the Figure is now EV5).

2. Fig 2 - are "expressed ratios" the best term for metabolite levels? Unlike genes, where such heat maps are often used, the metabolites are not 'expressed'. How about 'relative metabolite level' instead?

RESPONSE: Good point. The axis now reads “relative abundance”.

3. Page 8: "We also measured the MID values from the media of the same cultures used to prepare the cell extracts." Where are these data? We don't see them in File S2?

RESPONSE: The data are in File S2 (there are many ‘sheets’ in the file). In sheets 3,4 are the MID values and the analysis from metabolites in the media.

4. Fig 4B - the x-axis labeling is missing for the bat1- cells

RESPONSE: Corrected. Note that new DNA content measurements are now shown in Figure 3C.

5. Although the authors state repeatedly that they show "for the first time in any system" that TORC1 activity is dynamic in the cell cycle, similar observations have already been made before, for instance showing high mTORC1 activity in the G1/S transition in the Drosophila wing disc or low mTORC1 activity during mitosis in mammalian cells (see PMIDs 28829944, 28829945, and 31733992). The text should be amended accordingly.

RESPONSE: Thank you. Corrected.

6. There are two entries for valine in File S1/Sheet8. Why?

RESPONSE: The reason is that they were detected in both analytical pipelines (primary metabolites and biogenic amines; primary metabolites were measured with GC-TOF MS, while biogenic amines with HILIC-QTOF MS/MS), which were combined in the Table. We did not describe it adequately in the previous version. We do now, in the Methods. We also note that the raw data from each method are shown in the corresponding supplemental files. We combined them in the Table used in the Figure for display purposes. We also note that the amino acids were also measured by another method (PTH-based HPLC). Hopefully, the new edits in the Methods clarify these points.

Reviewer #1 (Significance (Required)):

Significance

Despite the well-known effects of pharmacological or genetic manipulations of TORC1/mTORC1 on cell cycle progression, whether and how mTORC1 activity itself is physiologically coupled to cell cycle progression is still an insufficiently studied aspect. Hence this study provides an interesting link between cell-cycle dependent regulation of amino acid biosynthesis and TORC1 regulation. Importantly, the results of this study rely on centrifugal elutriation to obtain cell cycle synchronization, thus ruling out potential metabolic artifacts due to pharmacological methods. The observed changes in metabolic flux are therefore likely genuine and represent the major strength of the study. The major limitation is the lack of strong evidence supporting the notion that the increase in Leu biosynthesis at late G1 or S-phase is causing the increase in TORC1 activity.

The major advance is conceptual - that amino acid biosynthesis rates are cell-cycle dependent.

These results will be of interest to a broad audience of people studying the cell cycle, cell growth, TORC1 activity, cell metabolism and cancer.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

This paper provides evidence that branched chain amino acid (BCAA) in the G1 phase of the cell cycle, fueled by pyruvate generated by glucose catabolism activates cell growth and allows cells to reach the critical size required for entry into S phase by activation of TORC1 signaling. Previous work had indicated that Leucine supplementation of a bat1 bat2 mutant, lacking both enzymes that catalyze BCAA from the alpha-keto acid precursors and starved on minimal medium, led to TORC1 activation. This work is significant in suggesting that BCAA synthesis from glucose is responsible for a cyclic activation of TORC1 necessary for a normal rate of cell growth in the G1 phase of the cell cycle.

The study employs metabolic flux analysis of metabolites derived from glucose following a pulse-chase with different isotopes of glucose in synchronized early G1 cells (obtained by elutriation) throughout one cell cycle. They claim that the only compelling changes in metabolites observed as the cell cycle proceeds was a decline in pyruvate containing only one heavy 13C carbon atom and a corresponding increase in Leu (M6) with 6 heavy carbon atoms, which is interpreted to indicate Leu synthesis from pyruvate that begins in early G1 and peaks at mitosis. They show that a bat1 mutant exhibits a slow-growth phenotype that can be mitigated only by valine (although they infer similar effects for Leu and Ile that I find unconvincing) and they observed reductions in all three BCAAs in different experiments that measure steady amino acid levels in different ways (although the results are compelling only for Val). They go on to show evidence that the bat1 mutation reduces birth and mean cell size and leads to an increased proportion of G1 cells in asynchronous cultures, and they claim that bat1 cells take much longer than WT to achieve the same size found when a synchronized WT culture reaches 50% budding (although they don't show the data for this last point.) Interestingly, they find that deleting BAT1 suppresses sensitivity to the TORC1 inhibitor rapamycin (Rap), consistent with the idea that the bat1 mutation impairs TORC1 activity in the same manner as Rap and that BCAA are required to activate TORC1 in WT cells to the level that can be impaired by Rap, as summarized in the model in Fig. 5F. Consistent with this, they present evidence that the bat1 mutation reduces TORC1 signaling as judged by diminished Rps6 phosphorylation (although it was not shown that this effect could be reversed by Val addition). They also show that TORC1 signaling/Rps6-P increases as the cell cycle progresses using elutriated early G1 cells, suggesting that TORC1 activity is periodic in the cell cycle (although they don't establish this periodicity through a second cell cycle).

General critique:
The conclusion that BCAA synthesis from glucose is responsible for a cyclic activation of TORC1 necessary for a normal rate of cell growth in the G1 phase of the cell cycle is potentially of considerable significance. There are however a number of puzzling aspects of the data that seem to weaken this conclusion. As described in greater detail below, it is difficult to explain why only Leu is synthesized from glucose during the cell cycle, and why only Val shows a marked reduction in the bat1 mutant that appears to be responsible for the slow-growth

phenotype. In addition, there are important controls lacking of showing that a Val supplement can suppress the G1 delay and reduction in TORC1 signaling in the bat1 mutant. In addition, the evidence that TORC1 activity is periodic in the cell cycle is lacking and it needs to be shown that Rps6-P levels are periodic through at least a second cell cycle.

Major comments:
-Why don't they observe synthesis of Ile and particularly Val in the metabolic flux experiment of Fig. 1, especially considering that only Val appears to be critically required for normal cell growth in the bat1 mutant based on the results in Fig. 3B?

RESPONSE: We now show the actual plots and the errors of all the measurements in Figure 2 (instead of a heatmap we had shown before). Valine (M5) levels show a very similar trend to leucine (M6). The variance in the measurements was higher, though, and statistically, the valine changes were less significant. Hence, it was more appropriate to highlight the leucine changes. Lastly, the new DNA content data (Figure 3C) show an effect upon the addition of leucine, albeit less significant than that of valine addition.

-The data in Fig. 3B do not show a convincing increase in growth of the bat1 mutant with addition of Leu and Ile; and the stimulation by Val alone seems identical to that seen with Val in combination with Leu and Ile. Thus, it appears that the slow-growth of the bat1 mutant results only from reduced Val levels, not all 3 BCAAs, which is at odds with their interpretation of the data.

RESPONSE. As mentioned above, the effect of valine is more pronounced than leucine's, but leucine does have consequences, best shown in the DNA content analysis (new Figure 3C). We also note that valine alone is insufficient to suppress the growth and cell cycle defects of bat1 cells. The latest data we have added (see Figures 3 and 5) are consistent with the interpretation that at least some de novo synthesis of BCAAs in the cell may be needed, explaining why exogenous BCAAs, including valine, are unable to correct the defects of bat1 cells fully.

-they claim to see reductions in all three BCAAs in the bat1 mutant; however, no significant reduction was found for Leu in Fig. EV3, and only Val was altered by the 1.5-fold cut-off imposed on the MS metabolomics data in Fig. EV4 (which could be appreciated only by an in-depth examination of the supplementary data in File S1-the Val, Leu, and Ile dots should be labeled in Fig. EV4). In addition, the reductions in Ala and Gly showin in Fig. EV3 were not found in the MS analysis of Fig. EV4. It needs to be acknowledged that the metabolomics data show a marked reduction in the bat1 mutant only for Valine with little or no change in Leucine levels. This result is difficult to explain with the simple models shown in Fig. 3A and 5F, which requires additional comment. The authors should acknowledge the much greater effect of the bat1 mutation on Val levels versus Leu and Ile, revealed both by measuring the levels of BCAAs in the mutant and comparing the BCAAs for rescuing the slow-growth of the mutant, and explain

how this can be reconciled with the results in Fig. 2 where only Leu and not Val or Ile synthesis was detected.

RESPONSE. The perceived discrepancy in the steady-state measurements could easily arise from the different analytical methods used in each case. The differences are less substantial than the reviewer implies. For steady-state measurements in BAT1 vs. bat1 cells, we used the PTH-based method (which only detects amino acids) and two different MS-based pipelines (which detect various metabolites). From the MS-based analyses, the drop for all BCAAs was statistically significant. Although the magnitude of the drop was greater for valine (about 60% for valine vs. ~30% for isoleucine and leucine). Why is this a problem?

As for the valine changes in the isotope tracing experiments, as we mentioned above, the trend for valine (M5) was similar to that of leucine (M6) (now, hopefully, that data is shown better in Figure 2). Furthermore, as we commented above (see response to Reviewer 1) and now stated in the text, our isotope tracing experiments measure only the rate of synthesis, which need not match the steady-state abundances. The latter are affected by a multitude of variables, including the turnover of proteins and amino acids, not to mention their partition into distinct intracellular pools.

Lastly, please note that we have now added PTH-based measurements of amino acid levels in the cell cycle of wild type cells (new Figure EV2). As mentioned in our response to Reviewer 1, we found that changes in the steady-state levels of BCAAs had a similar pattern, and those changes were most significant for valine (rising 30-40% from late G1 to G2/M).

-They need to add the data indicating that the bat1 mutant requires longer than WT cells to reach the ~35 fL volume at which 50% of WT cells are budded.

RESPONSE: We added all that data (new Figure EV6) and discussed it better in the text. Note that our elutriation analyses allow accurate estimates of the G1 duration, which is at least 2x longer in bat1 vs. BAT1 cells.

-It seems important to show that Val supplementation can suppress the overabundance of G1 cells in bat1 mutant cells shown in Fig. 4C; and can restore sensitivity to Rap and Rps6-P accumulation in bat1 mutant cells (in Fig.s 5A & B).

RESPONSE: Excellent suggestions. We now present the requested experiments. The DNA content data are in Figure 3C, and the phospho-Rps6 data in the new Figure 4D are discussed in the text. Briefly, exogenous valine, and to a lesser extent leucine, suppressed the G1 accumulation, but not to wild type levels. Exogenous valine also substantially increased TORC1 activity (>10-fold).

-It seems important to show that Rps6-P will decline in M phase and increase during a second cell cycle to establish that TORC1 activity actually fluctuates in the cell cycle instead of just by reduced by the manipulations involved in collecting young G1 cells by elutriation.

RESPONSE: The second cycle comment is not pertinent to our elutriation setup. The two-cycle approach should be used in arrest-and-release synchronizations to minimize arrest-related artifacts when cells continue to grow in size. This is why we used elutriation in the first place, as described in the text, to avoid such artifacts. In elutriations it is the first cycle, exclusively of daughter cells, that can be meaningfully scored. After that, the cells lose synchrony very fast because you have mothers (which grow in size very little) and daughters (which need to double in size until mitosis). Hence, the second cycle will be meaningless and impossible to interpret.

Reviewer #2 (Significance (Required)):

General Assessment:
Strengths: Evidence for BCAA biosynthesis from glucose in the G1 phase of the cell cycle, and evidence obtained from analyzing the bat1 mutant that BCAA synthesis underlies activation of TORC1 early in the cell cycle in a manner required to achieve the critical cell size necessary for G1 to S transition.
Weaknesses: Lack of evidence for Val biosynthesis in G1 despite evidence that Val limitation is more crucial than Leu limitation in the bat1 mutant; lack of confirmation that Val limitation underlies the delayed G1-S transition and reduced TORC1 signaling in the bat1 mutant; and lack of compelling evidence that TORC1 activity is periodic in WT cells.

Advance: This would be the first evidence that TORC1 activity varies through the cell cycle in a manner controlled by synthesis of BCAAs

Audience: This advance would be of great interest to a wide range of workers studying how the cell cycle is regulated and the role of TORC1 in controlling cell growth and division in normal cells and in human disease.

My expertise: Mechanisms of metabolic regulation of gene expression at the transcriptional and translational levels in budding yeast

Referees cross-commenting

Ref. #1's major comment 1 echoes my request for clarification about whether Leu, and not just Val, is limiting growth in the bat1 mutant, and also the need to determine which BCAA supplement to bat1 cells will restore TORC1 activity (which was also requested by Ref. #3). I agree with this reviewer's request to provide evidence that Leu levels actually increase during G1 progression (comment #2). I also think the suggested experiments in Comment #3 are reasonable for their potential to provide stronger evidence that Leu production in the G1 phase of wild-type cells activates TORC1, as currently the argument is based on the finding of low TORC1 activation in bat1 cells (that seem to be limiting for Val vs. Leu). Comment #4 echoes similar requests made by both me and Ref. #3. Ref. #3's major comments 1 and 3 mirror two of my major comments. I wasn't convinced of the need to monitor Sch9 versus Rps6 phosphorylation as a read-out of TORC1 activity-does being a direct substrate truly matter? Regarding comment 5, I wasn't convinced of the need to include Rap-sensitive or -resistant control strains for the analysis in Fig. 5A. And regarding comment 4, while it would be interesting to examine if TORC1 regulates BCAA synthesis during cell cycle progression, this seems to be outside the scope of a demonstration that BCAA synthesis stimulates TORC1.

Thus, it seems we all agree on certain experiments that need to be carried out, and Ref. #1 has rightly proposed a few others with the potential to strengthen the evidence that Leu production during G1 phase mediates cyclic activation of TORC1

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

In this manuscript, Blank and colleagues measure the synthesis of various metabolites from glucose during cell cycle progression and observe an increased synthesis of branched-chain amino acids (BCAA) from the early G1 to late G1 phase. Interestingly, they also found a gradual increase in TORC1 activity from the early G1 to the S phase which is proposed to be dependent on BCAA synthesis.

Major comments:

1. The authors show that TORC1 activity increases from the early G1 to the S phase. TORC1 activity is sensitive to short-term starvations caused during changing media or centrifugations. Hence, the concern arises regarding the increased pattern of TORC1 activity during the cell cycle. Is it really a biological phenomenon or a cellular adaptation to experimental conditions? Can authors provide more support for this observation? Can authors monitor the cell cycle for the two cell cycles to confirm that TORC1 activity shows a wavy pattern?

RESPONSE: The same point was also made by Reviewer #2. As we noted in our response above, "The second cycle comment is not pertinent to our elutriation setup. The two-cycle approach should be used in arrest-and-release synchronizations to minimize arrest-related artifacts when cells continue to grow in size. This is why we used elutriation in the first place, as described in the text, to avoid such artifacts. In elutriations it is the first cycle, exclusively of daughter cells, that can be meaningfully scored. After that, the cells lose synchrony very fast because you have mothers (which grow in size very little) and daughters (which need to double in size until mitosis). Hence, the second cycle will be meaningless and impossible to interpret."

2. The authors use Rps6 phosphorylation as a read-out of TORC1 activity, which is not a direct substrate of TORC1. Analysis of the direct substrates of TORC1, such as phosphorylation of Sch9 will solidify the author's claim.

RESPONSE: The reviewers discussed this point (see their comments above). We agree with the opinion that Rps6 phosphorylation accurately reports on TORC1 activity (also used in the fly experiments we now cite, as requested by Reviewer 1). For all our experiments' objectives and conclusions, it doesn't matter if the phosphorylation of Rps6 lies more downstream than Sch9 phosphorylation.

3. Authors show that Bat1 lacking strain have reduced TORC1 activity. Can authors restimulate these cells with Leucin, Valine, and Isoleucine individually or in combination to identify the critical amino acid for the TORC1 activity?

RESPONSE: Yes, that is an excellent suggestion. We show the experiment in Figure 4D (see previous response). Valine showed pronounced activation (>10-fold).

4. The authors claim that increased BCAA synthesis is necessary for TORC1 activation. Since TORC1 is shown to be upstream of amino acid biosynthesis pathways, it will be interesting to check if TORC1 per se regulates BCAA synthesis during cell cycle progression. The authors could inhibit TORC1 by rapamycin treatment and monitor if the BCAA synthesis still shows cell cycle-dependent modulation.

RESPONSE: The reviewers also discussed this point (see their comments above). We agree with the view that it is a very substantial undertaking, well beyond the scope of this work.

5. In Figure 5A, the use of any rapamycin-sensitive and rapamycin-resistant strains as controls will strengthen their claim of TORC1 inhibition being epistatic to Bat1 deletion, since the rapamycin in minimal media might be less effective.

RESPONSE: Again, the reviewers also discussed this point (see their comments above). We agree that it will not add much to the conclusions in the context of all the data we show and the existing literature.

Minor comments:
1. The data of metabolic labeling, especially various species M1, M2, M3, etc., of an individual metabolite is difficult to understand for the general readers. Hence, a schematic explaining various species might be helpful.

RESPONSE: We added a new Figure (EV1) delineating the carbons from glucose to valine and leucine.

2. Please describe the elutriation approach in more detail with media conditions and buffer conditions to understand the overall experimental setup.

RESPONSE: We now added this information (see the second section of the Materials and Methods).

Reviewer #3 (Significance (Required)):

Significance:
Overall, this study presents an interesting observation to the researchers working in TORC1 and cell cycle regulation.

1st Editorial Decision **12th May 2023**

Dear Dr. Polymenis,

Thank you for transferring your revised manuscript for consideration by EMBO reports via Review Commons. The three original referees who were asked to re-evaluate the revised version have now seen it, and we have received the full set of their reports, which are included below.

As you will see, referee #1 points out that the previously identified issues have been addressed, and they now recommend publication in EMBO reports. The other two referees agree that the findings are novel and interesting, but they also identify a few remaining limitations that should be addressed before the paper can be accepted. Given their constructive and detailed comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of another round of review, and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next version of the manuscript. If you have any questions or comments, we could also discuss the revisions in a video chat, if you like.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we usually recommend a revision within 3 months (August 11th). Please discuss with me the revision progress ahead of this time if you require more time to complete the revisions.

From the editorial side, there are also a few things that we need from you:

1. Please note that you can format your manuscript either as a Report or as an Article. For Reports, the manuscript should not exceed 27,000 characters (including spaces but excluding Materials & Methods and References; you currently have 24,930 characters) and 5 main plus 5 Expanded View figures. The Results and Discussion sections must be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For an Article there are no length limitations, but it should have more than 5 main figures and the Results and Discussion sections must be separate. In both cases, the entire Materials and Methods must be included in the main manuscript file.

2. Please submit a .docx formatted version of the revised manuscript text (including legends for main figures, EV figures and tables); please remove main and EV figures from the manuscript file. Individual production-quality figure files should be uploaded as .eps, .tif, .jpg (one file per figure). You can see our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages
https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3. We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content (including page numbers). Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:
<https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

4. Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in appropriate public databases (see <
https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Specifically, we would kindly ask you to provide public access to the following datasets/data:
- Mass spectrometry data.

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data availability " section (placed after Materials & Methods) that follows the model below (see also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>):

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note: all links should resolve to a page where the data can be accessed. ***

*** Note: the Data Availability Section is restricted to new primary data that are part of this study. ***

5. The legends of EV Figures should be provided at the end of the manuscript, following the main Figure legends.

6. The details of supplementary items should be removed from the manuscript text.

7. Please update your competing interests statement: the heading should be "Disclosure and competing interests statement". Please note that we request authors to consider both actual and perceived competing interest. Please review our policy (<https://www.embopress.org/competing-interests>).

8. The author contributions statement should be removed from the manuscript file. Instead, we now use CRediT to specify the contributions of each author in the journal submission system. Please use the free text box to provide more detailed descriptions. See also our guide to authors:
<https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>.

9. Please make sure that all corresponding authors have supplied their ORCID IDs.

10. We noticed that figure callouts for File S8 and S9 (which need to be renamed to Appendix Figure S#) are missing, please make sure that all panels are called out in your revised manuscript.

11. The legends of the EV Figures should remain in the manuscript (following the main Figures legends, at the end of the manuscript). Supplementary Files S1-S4 should be uploaded as Dataset EV1-EV4, and their callouts in the manuscript should be updated accordingly. Please include the legends of the EV Datasets in a separate sheet of each Excel file.

12. Supplementary Files S5-S9 need to be provided in the Appendix file and renamed to Appendix Figure S# etc. Please remember to update the callouts in the manuscript accordingly.

13. Please include a Table of Contents with page numbers on the first page of your Appendix. The figures included in the Appendix need to be renamed (e.g. to Appendix Figure S1 etc.). The table should be named Appendix Table S1.

14. Please note that EMBO press papers are accompanied online by:
A) a short (1-2 sentences) summary of the findings and their significance,
B) 2-4 short bullet points highlighting the key results, and
C) a synopsis image that is exactly 550 pixels wide and 300-600 pixels high (the height is variable). You can either show a model or key data in the synopsis image. Please note that the text needs to be readable at the final size.
Please upload this information along with your revised manuscript (the text for A and B should be provided in a separate Word file).

15. Our source data coordinator, Dr. Hannah Sonntag, has already contacted you with a list of Figure panels for which we request source data, along with useful advice and tips on how to organize and upload your data. Please do not hesitate to contact her directly (h.sonntag@source-data.org) if you have any questions about source data.

16. Please note that only 5 EV Figures can be typeset. The 6th main Figure could either be promoted to a main Figure (in case you decide to format your manuscript as an Article, which has no limitations in the number of main Figures) or otherwise it should be moved to the Appendix, renamed to an Appendix Figure S#.

17. Your Figure legends have been inspected by our data editors for completeness and accuracy. Please see the required changes in the attached Word file and address all comments in your revised manuscript (with tracked changes).

Please also note that as part of the EMBO publications' Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You can opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

We would also welcome the submission of cover suggestions or motifs to be used by our Graphics Illustrator in designing a cover.

We look forward to seeing a final version of your manuscript as soon as possible. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Ioannis Papaioannou, PhD
Editor
EMBO reports

Referee #1:

The authors have addressed the issues raised in the original review.

Referee #2:

The authors have improved the manuscript significantly by showing that supplementing bat1 cells with Val, but not Leu or Ile, stimulates TORC1-mediated phosphorylation of Rps6, which correlates with the ability of Val but not the other two BCAAs to improve the growth of bat1 cells. They have also pointed out their evidence that the rate of Val synthesis from glucose, and not only that of Leu synthesis, appears to increase during the G1/S phases; and also their evidence that steady-state Leu levels are significantly reduced in the bat1 mutant, albeit to a lesser degree than that seen for Val. The authors did not attempt to provide additional evidence that TORC1 activity is cyclic by measuring Rps6-P levels through two successive cell cycles, arguing in their Rebuttal that releasing synchronized cells from a cell cycle arrest would be required for this. They apparently believe that their experiment conducted using newly budded cells prepared by elutriation is the optimal approach, or at least sufficient, to draw the conclusion that TORC1 activity is cyclic through the cell cycle, and that the other approach they mentioned is superfluous. Not being an expert on cell cycle periodicity, I cannot dispute their conclusion.

However, I still object to the claim that "Exogenous supplementation of BCAAs in all combinations suppressed the growth defect of bat1Δ cells, especially when valine was present (Figure 3B)" as this figure shows no evidence of improved cell growth unless Val is supplemented, even for the combination of Leu/Ilv. It is conceivable that the combination of all three amino acids improves growth better than Val alone, but this would require additional evidence to be established. They attempted to bolster the claim that Leu can improve cell growth with new measurements of DNA content shown in Fig. 3C. These data support the stimulatory effect of Val on growth, which can now be attributed to a decrease in the duration of the G1 phase; however, running a t-test I found that the small effect of Leu in decreasing the %G1 cells is not quite statistically significant (P=0.07). They should try to bolster their claim that Leu and not only Val reduces the G1 phase by comparing the combination of Leu+Val, or all three BCAAs combined, to Val alone on %G1 phase cells with additional measurements of DNA content. If they chose not to do these experiments they should acknowledge that only Valine confers a significant improvement of cell growth and reduction in the G1 phase in the bat1 mutant (which is exactly what they observed for rescue of TORC1 activity in Fig. 4D), and add text offering an explanation for why Leu has little or no effect.

Referee #3:

In this revised manuscript, Blank and colleagues measure the synthesis of various metabolites from glucose during cell cycle progression and observe an increased synthesis of branched-chain amino acids (BCAA) from the early G1 to late G1 phase. Interestingly, they also found a gradual increase in TORC1 activity from the early G1 to the S phase which is proposed to be dependent on BCAA synthesis.

Major comments:

1. The authors show that TORC1 activity increases from the early G1 to the S phase. TORC1 activity is sensitive to short-term starvations caused during changing media or centrifugations. Hence, the concern arises regarding the increased pattern of TORC1 activity during the cell cycle. Is it really a biological phenomenon or a cellular adaptation to experimental conditions? Can authors provide more support for this observation? Can authors monitor the cell cycle for the two cell cycles to confirm that TORC1 activity shows a wavy pattern?

RESPONSE: The same point was also made by Reviewer #2. As we noted in our response above, "The second cycle comment is not pertinent to our elutriation setup. The two-cycle approach should be used in arrest-and-release synchronizations to minimize arrest-related artifacts when cells continue to grow in size. This is why we used elutriation in the first place, as described in the text, to avoid such artifacts. In elutriations it is the first cycle, exclusively of daughter cells, that can be meaningfully scored. After that, the cells lose synchrony very fast because you have mothers (which grow in size very little) and daughters (which need to double in size until mitosis). Hence, the second cycle will be meaningless and impossible to interpret."

Reviewer's Response: It is understandable that there are technical limitations. However, the concern regarding the cell cycle-dependent activation of TORC1 due to any artifacts in elutriation experiments remains unanswered. Are elutriation washing steps done with SMM media? Washing with PBS or water could reduce the TORC1 activity and lead to the downregulation of TORC1. If not done, can the authors revalidate the observation upon washing with SMM media and retaining nutrients throughout the experiment?
Is there any other alternative approach to support the cell cycle-dependent activation of TORC1? E.g., can cells in G1 and S phases be sorted by FACS and check whether they have a difference in TORC1 activity? Or test the second cycle of the arrest-and-release method to provide more support for this observation?

2. The authors use Rps6 phosphorylation as a read-out of TORC1 activity, which is not a direct substrate of TORC1. Analysis of the direct substrates of TORC1, such as phosphorylation of Sch9 will solidify the author's claim.

RESPONSE: The reviewers discussed this point (see their comments above). We agree with the opinion that Rps6 phosphorylation accurately reports on TORC1 activity (also used in the fly experiments we now cite, as requested by Reviewer 1). For all our experiments' objectives and conclusions, it doesn't matter if the phosphorylation of Rps6 lies more downstream than Sch9 phosphorylation.

Reviewer's Response: This does not satisfactorily answer the concern. Rps6 phosphorylation has been shown to be dependent on both TORC1 and TORC2 activity (Yerlikaya et al 2016, MBioC). Since, cell cycle-dependent TORC1 activation is the novel and interesting aspect of the present paper, solidifying this observation is necessary. The authors could any alternative read-outs such as Sch9, Atg8, Dot6 or any TORC1-transcriptional read-out to further support their interesting observation.

3. Authors show that Bat1 lacking strain have reduced TORC1 activity. Can authors restimulate these cells with Leucin, Valine, and Isoleucine individually or in combination to identify the critical amino acid for the TORC1 activity?

RESPONSE: Yes, that is an excellent suggestion. We show the experiment in Figure 4D (see previous response). Valine showed pronounced activation (>10-fold).

Reviewer's Response: It is interesting to see that Valine has a more pronounced effect than Leucine on TORC1 activation. However, in the current manuscript, initially, authors stress mainly on the increase in Leucine synthesis rather than Valine and then connect it to TORC1 activation by BCAA. Why? Can the authors reformat the text in the abstract and results section to highlight valine as well?

4. The authors claim that increased BCAA synthesis is necessary for TORC1 activation. Since TORC1 is shown to be upstream of amino acid biosynthesis pathways, it will be interesting to check if TORC1 per se regulates BCAA synthesis during cell cycle progression. The authors could inhibit TORC1 by rapamycin treatment and monitor if the BCAA synthesis still shows cell cycle-dependent modulation.

RESPONSE: The reviewers also discussed this point (see their comments above). We agree with the view that it is a very substantial undertaking, well beyond the scope of this work.

Reviewer's Response: OK

5. In Figure 5A, the use of any rapamycin-sensitive and rapamycin-resistant strains as controls will strengthen their claim of TORC1 inhibition being epistatic to Bat1 deletion, since the rapamycin in minimal media might be less effective.

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Reviewer's Response: OK

Minor comments:
1. The data of metabolic labeling, especially various species M1, M2, M3, etc., of an individual metabolite is difficult to understand for the general readers. Hence, a schematic explaining various species might be helpful.

RESPONSE: We added a new Figure (EV1) delineating the carbons from glucose to valine and leucine.

Reviewer's Response: OK

2. Please describe the elutriation approach in more detail with media conditions and buffer conditions to understand the overall experimental setup.

RESPONSE: We now added this information (see the second section of the Materials and Methods).

Reviewer's Response: Washing conditions were not clear.

Reviewer #3 (Significance (Required)):

Significance:
Overall, this study presents an interesting observation to the researchers working in TORC1 and cell cycle regulation.

We thank the reviewers for their comments and further suggestions. We have added more experiments and edited the text as needed to address their comments. Our detailed response to each point follows, shown in bold.

Referee #1:

The authors have addressed the issues raised in the original review.

Referee #2:

The authors have improved the manuscript significantly by showing that supplementing bat1 cells with Val, but not Leu or Ile, stimulates TORC1-mediated phosphorylation of Rps6, which correlates with the ability of Val but not the other two BCAAs to improve the growth of bat1 cells. They have also pointed out their evidence that the rate of Val synthesis from glucose, and not only that of Leu synthesis, appears to increase during the G1/S phases; and also their evidence that steady-state Leu levels are significantly reduced in the bat1 mutant, albeit to a lesser degree than that seen for Val. The authors did not attempt to provide additional evidence that TORC1 activity is cyclic by measuring Rps6-P levels through two successive cell cycles, arguing in their Rebuttal that releasing synchronized cells from a cell cycle arrest would be required for this. They apparently believe that their experiment conducted using newly budded cells prepared by elutriation is the optimal approach, or at least sufficient, to draw the conclusion that TORC1 activity is cyclic through the cell cycle, and that the other approach they mentioned is superfluous. Not being an expert on cell cycle periodicity, I cannot dispute their conclusion.

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additional measurements of DNA content. If they chose not to do these experiments they should acknowledge that only Valine confers a significant improvement of cell growth and reduction in the G1 phase in the bat1 mutant (which is exactly what they observed for rescue of TORC1 activity in Fig. 4D), and add text offering an explanation for why Leu has little or no effect.

AUTHORS NEW RESPONSE: As we describe in the manuscript, even when all 3 BCAAs are added, there is no complete suppression of the bat1 growth phenotype (see Figure 3). Similar results were also observed for the bat1,2 double mutants by Kingsbury et al. As for the t-test the reviewer performed, a simple t-test for n=3 is inappropriate because of normality uncertainties/violations. Instead, we performed the bootstrap version, which employs resampling and randomization, and the associated p-value was p=0.023. We now provide this information in the text. We agree with the reviewer that valine shows the highest degree of suppression, and have stated that in the text. As for an explanation for the lesser ability of Leu to suppress, we do not have any to offer.

Referee #3:

In this revised manuscript, Blank and colleagues measure the synthesis of various metabolites from glucose during cell cycle progression and observe an increased synthesis of branched-chain amino acids (BCAA) from the early G1 to late G1 phase. Interestingly, they also found a gradual increase in TORC1 activity from the early G1 to the S phase which is proposed to be dependent on BCAA synthesis.

Major comments:

1. The authors show that TORC1 activity increases from the early G1 to the S phase. TORC1 activity is sensitive to short-term starvations caused during changing media or centrifugations. Hence, the concern arises regarding the increased pattern of TORC1 activity during the cell cycle. Is it really a biological phenomenon or a cellular adaptation to experimental conditions? Can authors provide more support for this observation? Can authors monitor the cell cycle for the two cell cycles to confirm that TORC1 activity shows a wavy pattern?

RESPONSE: The same point was also made by Reviewer #2. As we noted in our response above, "The second cycle comment is not pertinent to our elutriation setup. The two-cycle approach should be used in arrest-and-release synchronizations to minimize arrest-related artifacts when cells continue to grow in size. This is why we used elutriation in the first place, as described in the text, to avoid such artifacts. In elutriations it is the first cycle, exclusively of daughter cells, that can be meaningfully scored. After that, the cells lose

synchrony very fast because you have mothers (which grow in size very little) and daughters (which need to double in size until mitosis). Hence, the second cycle will be meaningless and impossible to interpret."

Reviewer's Response: It is understandable that there are technical limitations. However, the concern regarding the cell cycle-dependent activation of TORC1 due to any artifacts in elutriation experiments remains unanswered. Are elutriation washing steps done with SMM media? Washing with PBS or water could reduce the TORC1 activity and lead to the downregulation of TORC1. If not done, can the authors revalidate the observation upon washing with SMM media and retaining nutrients throughout the experiment?

AUTHORS NEW RESPONSE: That is precisely how we do all our experiments. There are no washes with buffers (PBS, etc.). The cells are continuously in the culture medium (synthetic minimal). We now clarify this in the text.

Is there any other alternative approach to support the cell cycle-dependent activation of TORC1? E.g., can cells in G1 and S phases be sorted by FACS and check whether they have a difference in TORC1 activity? Or test the second cycle of the arrest-and-release method to provide more support for this observation?

AUTHORS NEW RESPONSE: We understand the reviewer's view, but we feel strongly that we have used the most demanding and best-suited synchronization method for the objectives of this study, for the following reasons:

1. **Elutriation perturbs cellular physiology the least compared to any of the methods suggested by the reviewer.**
2. **Elutriation also provides the highest resolution of the G1 phase.**
3. **As we and others have detailed in the literature (cited in the manuscript), arrest-and-release methods do not properly probe links between growth and division, especially for G1 effects. During the arrest, the cells continue to grow, fulfilling their growth requirements for division, and these effects are carried over to subsequent cell cycles.**
4. **As for FACS sorting, it is unclear what the reviewer is suggesting. How exactly would it be done?**
    a. **Assuming it would be on fixed cells (so DNA content can be measured) we are unaware of any phosphorylation queries after prolonged ethanol fixation and RNAse-treatment (required for DNA content measurements). Furthermore,**
        i. **How would one set the gates (1N -lumping all G1 cells together vs. 2N -essentially eliminating all S cells with intermediate DNA content vs. S cells between 1N and 2N)?**

ii. **The resolution in all the above cases would be poor (especially for yeast with its small genome, with closely spaced 1N and 2N peaks). Also, the G1 cells would 'look' the same, but clearly, there are differences in the phosphorylation of Rps6 as cells progress through G1.**

b. **If the reviewer had in mind some way to sort live cells (ignoring here the complex engineering needed to generate the appropriately fluorescent strains; a FUCCI indicator system is not developed in yeast), what markers would one use for sorting, not just G1 vs. S vs. M, but, say, to compare live early G1 vs. mid- vs. late G1 cells?**

c. **Even if all those problems are solved, the transit time to the instrument, the buffer changes, storage of cells on ice, etc., could introduce many more potentially problematic variables.**

Why would any of these approaches be superior to monitoring cell size and budding we report, of prototrophic, un-engineered cells that progress in their cell cycle, never arrested and without any buffer changes?

2. The authors use Rps6 phosphorylation as a read-out of TORC1 activity, which is not a direct substrate of TORC1. Analysis of the direct substrates of TORC1, such as phosphorylation of Sch9 will solidify the author's claim.

RESPONSE: The reviewers discussed this point (see their comments above). We agree with the opinion that Rps6 phosphorylation accurately reports on TORC1 activity (also used in the fly experiments we now cite, as requested by Reviewer 1). For all our experiments' objectives and conclusions, it doesn't matter if the phosphorylation of Rps6 lies more downstream than Sch9 phosphorylation.

Reviewer's Response: This does not satisfactorily answer the concern. Rps6 phosphorylation has been shown to be dependent on both TORC1 and TORC2 activity (Yerlikaya et al 2016, MBioC). Since, cell cycle-dependent TORC1 activation is the novel and interesting aspect of the present paper, solidifying this observation is necessary. The authors could any alternative read-outs such as Sch9, Atg8, Dot6 or any TORC1-transcriptional read-out to further support their interesting observation.

AUTHORS NEW RESPONSE: As suggested by the reviewer, we now include more data (see new Appendix Figure S2) using a previously described antibody targeting Sch9-pT737, as described by Kingsbury et al. We first confirmed that the signal is sensitive to rapamycin treatment. Then, we performed two separate elutriation experiments using this independent reagent. The pattern was very similar to what we observed for the anti-pRps6 antibody. Notably, this pattern was also evident in the prototrophic strain X2180-5A

(S288C background). Hence, looking at two outputs (Rps6 or Sch9) in two quite different backgrounds (CEN.PK or S288C), the evidence unambiguously points to dynamic changes in TORC1 activity in the cell cycle.

3. Authors show that Bat1 lacking strain have reduced TORC1 activity. Can authors restimulate these cells with Leucin, Valine, and Isoleucine individually or in combination to identify the critical amino acid for the TORC1 activity?

RESPONSE: Yes, that is an excellent suggestion. We show the experiment in Figure 4D (see previous response). Valine showed pronounced activation (>10-fold).

Reviewer's Response: It is interesting to see that Valine has a more pronounced effect than Leucine on TORC1 activation. However, in the current manuscript, initially, authors stress mainly on the increase in Leucine synthesis rather than Valine and then connect it to TORC1 activation by BCAA. Why? Can the authors reformat the text in the abstract and results section to highlight valine as well?

AUTHORS NEW RESPONSE: Done.

4. The authors claim that increased BCAA synthesis is necessary for TORC1 activation. Since TORC1 is shown to be upstream of amino acid biosynthesis pathways, it will be interesting to check if TORC1 per se regulates BCAA synthesis during cell cycle progression. The authors could inhibit TORC1 by rapamycin treatment and monitor if the BCAA synthesis still shows cell cycle-dependent modulation.

RESPONSE: The reviewers also discussed this point (see their comments above). We agree with the view that it is a very substantial undertaking, well beyond the scope of this work.

Reviewer's Response: OK

5. In Figure 5A, the use of any rapamycin-sensitive and rapamycin-resistant strains as controls will strengthen their claim of TORC1 inhibition being epistatic to Bat1 deletion, since the rapamycin in minimal media might be less effective.

RESPONSE: Again, the reviewers also discussed this point (see their comments above). We agree that it will not add much to the conclusions in the context of all the data we show and the existing literature.

Reviewer's Response: OK

Minor comments:
1. The data of metabolic labeling, especially various species M1, M2, M3, etc., of an individual metabolite is difficult to understand for the general readers. Hence, a schematic explaining various species might be helpful.

RESPONSE: We added a new Figure (EV1) delineating the carbons from glucose to valine and leucine.

Reviewer's Response: OK

2. Please describe the elutriation approach in more detail with media conditions and buffer conditions to understand the overall experimental setup.

RESPONSE: We now added this information (see the second section of the Materials and Methods).

Reviewer's Response: Washing conditions were not clear.

AUTHORS NEW RESPONSE: We now clarify this in the text. As we mentioned above, there are no washes with buffers. The cells are continuously in the culture medium (synthetic minimal).

Reviewer #3 (Significance (Required)):

Significance:
Overall, this study presents an interesting observation to the researchers working in TORC1 and cell cycle regulation.

Dear Michael,

Thank you for the submission of your revised manuscript to EMBO reports and for addressing our previous editorial requests. We have now received the reports of the two referees that were asked to re-evaluate your revised manuscript. Please find their comments below.

As you will see, there is only one remaining issue (mentioned by referee #2) which we need you to address before acceptance of your manuscript.

From the editorial side, there are also a few things that we need from you:

- Please note that the Abstract should be written in present tense.

- Please reformat your Table EV1 (that lists various reagents and materials) as a "Reagents and Tools Table" by filling one of the templates (Excel or Word template) that you can find in our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide ; under Materials and Methods --> Structured Methods). When you are ready to re-submit your manuscript, please upload this table to our manuscript tracking system as a "Reagent Table" file. The rest of your previous materials and methods section should follow under the heading "Methods and Protocols" (no other changes in your existing text are necessary).

- Please note that we need the ORCID IDs of all co-corresponding authors (no ID has been supplied for Karsten Hiller yet).

- We have previously asked you to address a number of requests of our data editors for completeness and accuracy of your Figure legends, using tracked changes. Unfortunately, tracked changes are not shown in your revised file, and it seems that not all of their comments have been addressed. Please check them all again and make sure that they are all addressed in your revised manuscript.

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We would also welcome the submission of cover suggestions or motifs to be used by our Graphics Illustrator in designing a cover.

We look forward to seeing a final version of your manuscript as soon as possible. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Best regards,

Ioannis

Ioannis Papaioannou, PhD
Editor
EMBO reports

Referee #2:

I still contend that their sentence on p. 9 "Exogenous supplementation of BCAAs in all combinations suppressed the growth defect of bat1Δ cells, especially when valine was present (Figure 3B), in agreement with a proposal that valine synthesis in yeast is primarily a mitochondrial process regulated by Bat1 (Takpho et al, 2018)." does not accurately describe the results in Fig, 3B where supplementation with Ile, Leu, or Ile+Leu confers no better growth of the bat1 mutant compared to mock.

Referee #3:

The authors have addressed the queries satisfactorily.

July 5, 2023
Dr. Ioannis Papaioannou
Editor, *EMBO Reports*

Dear Dr. Papaioannou,

We submit another revision of our manuscript (EMBOR-2023-57372) entitled "**Branched chain amino acid synthesis is coupled to TOR activation early in the cell cycle in yeast**" to EMBO Reports.

Below, I list the answers to the last remaining issues you outlined in your previous letter.

Thank you for your handling of our manuscript.

Sincerely,

Michael Polymenis

Referee #2

I still contend that their sentence on p. 9 "Exogenous supplementation of BCAAs in all combinations suppressed the growth defect of bat1Δ cells, especially when valine was present (Figure 3B), in agreement with a proposal that valine synthesis in yeast is primarily a mitochondrial process regulated by Bat1 (Takpho et al, 2018)." does not accurately describe the results in Fig, 3B where supplementation with Ile, Leu, or Ile+Leu confers no better growth of the bat1 mutant compared to mock.

RESPONSE: We now corrected this. The text reads: "Exogenous supplementation of BCAAs suppressed the growth defect of *bat1Δ* cells, mostly when valine was present (Figure 3B), …".

Editorial

- Please note that the Abstract should be written in present tense.

RESPONSE: Done

- Please reformat your Table EV1 (that lists various reagents and materials) as a "Reagents and Tools Table" by filling one of the templates (Excel or Word template) that you can find in our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide ; under Materials and Methods --> Structured Methods). When you are ready to re-submit your manuscript, please upload this table to our manuscript tracking system as a "Reagent Table" file. The rest of your previous materials and methods section should follow under the heading "Methods and Protocols" (no other changes in your existing text are necessary).

RESPONSE: Done

- Please note that we need the ORCID IDs of all co-corresponding authors (no ID has been supplied for Karsten Hiller yet).

RESPONSE: I had sent it as an email because somehow I couldn't input it on the site. His ORCID is: 0000-0001-9322-5820

- We have previously asked you to address a number of requests of our data editors for completeness and accuracy of your Figure legends, using tracked changes. Unfortunately, tracked changes are not shown in your revised file, and it seems that not all of their comments have been addressed. Please check them all again and make sure that they are all addressed in your revised manuscript.

RESPONSE: I apologize. I do not normally use Word, and I had missed some of the comments. I found the comments (from Vivian Killet, on May 03). I now highlight in the manuscript text file the edits, and I also list my response to the comments here:

> FIGURE 1: B and C (and also FIGURE 4 D) are of low resolution so that individual datapoints aren't easy to be seen.
>
> **RESPONSE: The new images I have uploaded are of higher resolution. I can see the datapoints.**
>
> FIGURE 1, 4, EV3, EV5: Please define the central band, boxes and whiskers of the boxplots. Please define the number and the nature, i.e. biological or technical, of the replicates.
>
> **RESPONSE: I have now added the following: "Each box is drawn from the first to the third quartile, with a horizontal line drawn in the middle to denote the median. The whiskers show the interquartile range (IQR), and they were drawn at 1.5xIQR. The replicates were all biological ones."**

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RESPONSE: I do not wish to opt out.

Michael Polymenis
Texas A&M University
Biochemistry and Biophysics
2128 TAMU
College Station, orcid||||||| 77843
United States

Dear Michael,

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

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Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Best regards,

Ioannis

Ioannis Papaioannou, PhD
Editor
EMBO reports

****

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Corresponding Author Name: Michael Polymenis
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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions

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 χ2 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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Data Availability Section

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods, Reagents and Tools Table

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Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Reagents and Tools Table

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Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Yes	Reagents and Tools Table, Materials and Methods

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Materials and Methods

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Yes	Materials and Methods

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Materials and Methods
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	There was no randomization procedure.
Include a statement about **blinding** even if no blinding was done.	Not Applicable	No blinding was done.
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Yes	Materials and Methods
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods. As we describe, we used non-parametric and robust bootstrap ANOVA tests.

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Materials and Methods, Figures
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Materials and Methods

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
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If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
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Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Not Applicable	
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Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	