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Translational control of lipogenic enzymes in the cell cycle of synchronous, growing yeast cells

Heidi Blank, Ricardo Perez, Chong He, Nairita Maitra, Richard Metz, Joshua Hill, Yuhong Lin, Charles Johnson, Vytas Bankaitis, Brian Kennedy, Rodolfo Aramayo and Michael Polymenis

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Transaction Report:

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

1st Editorial Decision 20 July 2016

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see from the reports, the referees overall express interest in the findings reported in your manuscript - especially the value in studying cell cycle-dependent changes in gene expression in a system unperturbed by drug treatment. However, you will see that they also raise a number of major and minor concerns that will have to be addressed before they can support publication of your manuscript in The EMBO Journal. This is most clearly seen in the comments from ref #2 who finds that additional mechanistic and functional insight on the consequences of increased Acc1 expression is required.

Given the referees' overall positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers in full. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance or rejection of your manuscript will therefore depend on the completeness of your responses in this revised version.

For the revised manuscript I would particularly ask you to focus your efforts on the following points:
-> Please extend the gene expression analysis beyond a single round of cell division as requested by both ref #1 and #2 to demonstrate that the alterations seen reflect cycling behaviour
-> Please address the comments from ref #2 in full, both with regard to overlap with earlier reports on Acc1 and for the functional contribution of cell cycle-dependent translational control to lipid biogenesis and membrane formation. The revised manuscript would need to have strong support

from ref #2 for us to take further steps towards publication.

-> In addition, I consulted with an additional technical advisor for the ribosome profiling analysis conducted and this person returned the following remarks:

'Regarding the request to evaluate the technical competency of this paper, I think the authors have made an effort to characterize the changes in translation with growth. However, one caveat of their approach is that they have used polyA+ to quantify mRNA levels in order to normalize the ribosome footprints and calculate translation efficiency. However, how do the authors know that changes in translation efficiency are not instead increase or decrease in the polyA+ mRNA population rather than changes in translation per se? '

Since this is a point that could potentially undermine several of the main conclusions drawn here I would ask you to reconsider the normalization strategy and extend it with additional analysis.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://emboj.embopress.org/about#Transparent_Process

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

REFEREE REPORTS

Referee #1:

The authors monitor translation efficiency across the cell cycle in synchronised yeast cells using ribosome profiling. Although similar experiments have been performed in mammalian cells, yeast has the unique advantage that cell cycle synchronisation can be performed without artefactually affecting cell size. Therefore, this study is novel and addresses an important biological question.

Overall this is an excellent piece of work. The experiments are carefully designed and executed, and the presentation is very clear.

I only have one concern about the data and conclusions. In all experiments the authors follow synchronised cell cultures for a single cell cycle (understandable, of course, given the complexity of the experiments). It is theoretically possible that the elevation in translation levels of some genes in G2 is due to an effect of elutriation on cells, and that it does not represent periodic behaviour (i.e., the levels of translation and proteins would remain high in subsequent cell cycles). This could be very easily addressed by the authors by following, for example, Acc1-TAP levels for two cell cycles. One would expect a decrease in levels as the cells enter the second cell cycle. The experiment could be done at low resolution (i.e., with fewer time points).

Minor points:
1. Does the ribosome profiling data show translation of the uORF in ACC1? If so, does the ribosomal density of the uORF usage change compared to that of the coding sequence?
2. Although very unlikely given the ribosome profiling data, it is formally possible that changes in protein abundance are due to alterations in protein stability. The authors should discuss this possibility (note that I do not think additional experiments are necessary).

PS. An important technical point that may be raised: The authors treated cells with cycloheximide (CHX) just before collection. Although CHX has been reported to affect the specific codons at which ribosome arrest, it is not thought to affect transcript-wide measurements of ribosome density. As the authors did the latter, the use of CHX in this work is completely appropriate.

Referee #2:

In this study, the authors use ribosome profiling to probe mRNA translational activities in synchronous cell populations. This analysis identified seventeen messages subjected to translational control and among those, three key enzymes in the de novo fatty acid (FA) synthesis pathway (ACC1, FAS1 and FAS2). The authors show that Acc1p protein levels are regulated by an upstream open reading frame (uORF) in a cell cycle- and nutrient dependent manner. When grown in a non-fermentable carbon source and in the absence of the ACC1-uORF, Acc1p levels are significantly upregulated and this correlates with a smaller cell size. Finally, although loss of the ACC1-uORF does not affect replicative life span, it does suppress the longer file span of the yeast S6 kinase homologue mutant, sch9.

General comments:
The study is generally well written; it applies a challenging methodology and makes the remarkable finding that FA synthesis is under translational control during the cell cycle (although this needs to be substantiated further - see below). The relatively small number of hits and the obvious close functional link between Acc1 and Fas, strongly support a coordinate increase in de novo FA sytnthesis late in the cell cycle. However, there is considerable previous work showing that Acc1 and FA synthesis are tightly regulated at many levels, including nutrient signals through AMPK, and that this is linked to cell cycle progression at G2/M and membrane homeostasis. The translational regulation reported here (actually the presence of the conserved uORF ACC1 has been previously reported in Cvijovic et al, BMC Bioinformatics 2007 8:295, but this is not mentioned in the manuscript) extends these studies but fails to address key questions arising from this observation and which in my view would be required for publication in EMBO J. Does the temporal increase in "lipogenic" enzymes drive membrane mass and cell size through phospholipid synthesis or FAs are channeled to triglycerides for storage in lipid droplets? In fact, recent studies in fission and budding yeast have documented an increase in droplets and triglycerides at later stages of the cell cycle, and it is known that hyperactive Acc1 alleles drive FAs to droplets without affecting phospholipids. How does this relate to the smaller size of cells with elevated Acc1p levels? Rather than exploring the mechanistic role of the Acc1 regulation during the cell cycle, the authors report a link to replicative lifespan, which is certainly interesting but left without an explanation, making its connection with the rest of the manuscript quite tenuous. Overall I find the data interesting but too preliminary at this stage for publication.

Other points:
I find that the timing of the Acc1p/Fas1p upregulation is not convincingly established; from the data in Figure 4, it is not clear to me when Acc1- and Fas-TAP protein levels peek. In Fig. 3B, the most significant increase takes place after 60 fL but the TAP-fusion sampling in Fig. 4 stop around 50fL, with a major increase between late 30s to 45 fL - which is not "late in the cell cycle" (l. 175).

With respect to the experimental setup for sample collection (i.e. allowing each separate cell population to reach a set cell size before harvesting), I do not think the authors can talk about "periodicity" in Acc1p levels; that would require comparing samples for one division post-elutriation.

Referee #3:

This manuscript by H.M. Blank et al. investigates how the levels of individual proteins vary through the cell cycle of the yeast Saccharomyces cerevisiae. For a decade or two, numerous reports have appeared showing the variations in specific mRNAs at different cell-cycle stages, but these reports have contributed only marginally to understand the mechanisms coordinating cell growth and cell-cycle progression. In this manuscript, the translational activities of individual mRNAs as well as the levels of specific proteins at different points in the cell cycle have been measured. This, together with the use of centrifugal elutriation to obtain a population of synchronous cells that have not been exposed to much stress, makes this study important and significant. In most previous reports using synchronously growing cells, stress has been employed to arrest the cells at a particular stage (temperature or drugs to arrest cell-cycle progression), and these methods have been shown to create artifacts and unwanted stress responses that perturb the cell cycle. It is self-evident that in a culture

in balanced growth, the cells at any particular stage in the cell cycle are, in principle, of the same size and have the same contents of macromolecules - otherwise the system is not in steady state. But the molecular mechanisms governing this steady state are not known. In this work the authors show that towards the end of a minimally perturbed cell cycle, in late G2, mRNAs related to lipogenesis are preferentially translated, maybe giving the first clues to the elusive coupling mechanism. These data were accumulated by doing ribosome profiling of the RNA extracted from cells emanating synchronously from the unbudded state in early G1 phase.
Although the method of centrifugal elutriation has the great advantage of producing cells that are little affected by the treatment itself, as opposed to other methods creating synchronous populations, it has the drawback of giving a lower degree of synchrony. This can be exemplified by Figure 1C, where the level of certain mRNAs' attachment to ribosomes is plotted as a function of cell size. It is not absolutely clear, but it seems that the 2- to 3-fold increase observed for CLN1 and CLN2 appear over a fairly large size range (from 45 to 55 fL). The uncertainty is manifested partly because of another problem, namely that high numbers of cells are required to perform the ribosome profiling experiment, and enough cells cannot be collected to perform a time series of one particular culture. Therefore, parallel samples were used and the assumption is that they are all similar. The alternative is to become "greedy" when same-size cells are collected, resulting in a wider variability is cell sizes and therefore lowered synchrony. It seems that the reviewers have made wise decisions and trade-offs to create data that can be interpreted with confidence. An indication of the degree of synchrony, such as a measurement of the kinetics of S phase entry and progression by flow cytometry, might be helpful.

The approach is novel and the results are interesting. The conclusions are well supported by the data presented. The manuscript is well written and relatively easy to understand.

Some specific comments, in the order of appearance in the manuscript:
1. Line 1. I am not sure why "growing" should be in the title. Maybe even "synchronous" should be deleted? "Translational control of lipogenic enzymes in the cell cycle of yeast cells" is perfectly OK.
2. Line 39. The statement that cell growth depends on protein synthesis appears rather obvious; I am not sure which part of Mitchison's work is referred to.
3. Line 43. May link up with the previous: It is pretty obvious that larger cells produce more proteins than small cells. And it does not follow from the statement before, that cell size is increasing exponentially through the cell cycle. Some stringency is required for these statements to become meaningful.
4. Line 105. An indication of how good the synchrony is, would be good to have. This will add confidence to the data and help their interpretation. It is possible that the synchrony is very good, which might suggest that an improvement in method might give better data and sharper changes in gene expression.
5. Line 225. It is said that all cell-cycle phases are increased in length, but they have not been measured. What is shown is that G1 and G2/M are increased, but nothing is known about S phase or G2 and M individually. Should be modified.
6. Line 260. There will probably always be some kind of coupling between growth and cell division. The point here is that the NORMAL coupling that occurs in unperturbed cultures is likely to be maintained.
7. Line 266. This line is a bit cryptic; the contents should be spelled out more clearly.
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9. Line 367. It would be helpful to indicate which chamber was used. This centrifuge does have rather large chambers, and it would be good to know that the largest one was used. Otherwise, the method can be improved.
10. Figure 6. If this is a culture growing in steady state the growth rates given in panel A should correspond to the specific growth rates plotted in panel C, bottom. The exact values are not clear, but it does not seem that the numbers correspond very well. This should be addressed. Futhermore, the p values are given above the line connecting the data sets for the two strains, as if the "distance" between the strains is the subject. If it is the difference in the ordinate values that is the matter, the notation should be changed.

RESPONSE TO REVIEWS (RE: EMBOJ-2016-95050)

We wish to thank the reviewers for their constructive suggestions as to how to improve our manuscript. As outlined below, we have responded positively and comprehensively to their criticisms, and have addressed all the points raised by each of the 4 referees. These responses include substantial new experimentation and revision of text. Our itemized responses to the reviewers' comments are as follows:

Referee #1:

The authors monitor translation efficiency across the cell cycle in synchronised yeast cells using ribosome profiling. Although similar experiments have been performed in mammalian cells, yeast has the unique advantage that cell cycle synchronisation can be performed without artefactually affecting cell size. Therefore, this study is novel and addresses an important biological question. Overall this is an excellent piece of work. The experiments are carefully designed and executed, and the presentation is very clear.

I only have one concern about the data and conclusions. In all experiments the authors follow synchronised cell cultures for a single cell cycle (understandable, of course, given the complexity of the experiments). It is theoretically possible that the elevation in translation levels of some genes in G2 is due to an effect of elutriation on cells, and that it does not represent periodic behaviour (i.e., the levels of translation and proteins would remain high in subsequent cell cycles). This could be very easily addressed by the authors by following, for example, Acc1-TAP levels for two cell cycles. One would expect a decrease in levels as the cells enter the second cell cycle. The experiment could be done at low resolution (i.e., with fewer time points).

RESPONSE: We have now added this experiment (see new Figure EV2), for more extended periods of time as cells entered a second cell cycle. We also added the following text (line 185): "The mitotic peak of Acc1p-TAP levels was not due to trivial technical reasons arising from elutriation artifact, because when cells were allowed to re-enter a second cell cycle Acc1p-TAP levels dropped substantially in the subsequent G1 phase (Fig. EV2)."

Minor points:

1. Does the ribosome profiling data show translation of the uORF in ACC1? If so, does the ribosomal density of the uORF usage change compared to that of the coding sequence?

RESPONSE: Yes it does. We now show these data in the new Figure EV4), and we added the following text (line 199): "We also noticed that ribosome footprint reads were present at the ACC1 uORF, and in higher numbers during the G1 phase of the cell cycle (Fig. EV4)."

2. Although very unlikely given the ribosome profiling data, it is formally possible that changes in protein abundance are due to alterations in protein stability. The authors should discuss this possibility (note that I do not think additional experiments are necessary).

RESPONSE: We have now added the following in the revised text (line 218): "Since both the wild type and the uORF mutant strain express the same Acc1p-TAP protein, differences in the levels of Acc1p-TAP between these strains in the cell cycle and in different nutrients are due to differential synthesis and not a result of altered protein stability."

PS. An important technical point that may be raised: The authors treated cells with cycloheximide (CHX) just before collection. Although CHX has been reported to affect the specific codons at which ribosome arrest, it is not thought to affect transcript-wide measurements of ribosome density. As the authors did the latter, the use of CHX in this work is completely appropriate.

RESPONSE: We agree and added the following statement (lines 401): "Although cycloheximide has been reported to affect the specific codons at which ribosomes arrest (Hussmann et al., 2015), it is not thought to affect transcript-wide measurements of ribosome density, which is the output we queried in this study."

Referee #2 (including further clarifications/comments from the referee obtained at a later time): In this study, the authors use ribosome profiling to probe mRNA translational activities in synchronous cell populations. This analysis identified seventeen messages subjected to translational control and among those, three key enzymes in the de novo fatty acid (FA) synthesis pathway (ACC1, FAS1 and FAS2). The authors show that Acc1p protein levels are regulated by an upstream open reading frame (uORF) in a cell cycle- and nutrient dependent manner. When grown in a non-fermentable carbon source and in the absence of the ACC1-uORF, Acc1p levels are significantly upregulated and this correlates with a smaller cell size. Finally, although loss of the ACC1-uORF does not affect replicative life span, it does suppress the longer file span of the yeast S6 kinase homologue mutant, sch9.

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RESPONSE: We added the Cvijovic et al reference (line 194).

Does the temporal increase in "lipogenic" enzymes drive membrane mass and cell size through phospholipid synthesis or FAs are channeled to triglycerides for storage in lipid droplets? In fact, recent studies in fission and budding yeast [see Yang et al (2016) MBoC 27, 2368 "Lipid droplets maintain lipid homeostasis during anaphase for efficient cell separation in budding yeast"] have documented an increase in droplets and triglycerides at later stages of the cell cycle, and it is known that hyperactive Acc1 alleles drive FAs to droplets without affecting phospholipids. Several studies have documented an increase in TAGs and LD size upon exit of budding yeast cells from logarithmic growth (see Kohlwein et al, (2013) Genetics 193, 1). This is relevant here because the uORF regulates Acc1p levels in response to nutrient availability and more specifically to a non-fermentable carbon source (glycerol), a condition that mimics glucose exhaustion during exit from log phase (diauxic shift) and which is known to regulate Acc1 activity and TAG metabolism (Hedbacker, K., and Carlson, M. (2008) Front. Biosci. 13, 2408; Hofbauer et al, (2014) Dev Cell 29, 729). My main criticism is that, despite several statements on "lipogenesis" throughout the manuscript, this evidence is missing. Therefore, I would like to see how phospholipids and TAGs, the two natural sinks of FAs, change in the uORF Acc1 mutant in cycling cells. Moreover, because, as explained above, in addition to cycling cells Acc1p is also regulated at the level of the exit of log growth (the "poor" carbon source experiment), phospholipid/TAG analysis should be also performed in the glycerol grown samples.

RESPONSE: We appreciate the comments by the reviewer and the follow-up clarifications that were provided. We were not aware of the Yang et al paper (it appeared after our submission), which looked at lipid droplets in cycling cells and it is pertinent to our study. Hence we used their methodology to quantify lipid droplet formation in M phase, in wild type and ACC1 uORF mutant cells in glucose media (new Figure EV6). We also quantified TAGs in glycerol as the reviewer suggested (new Figure EV7). From the same samples in each case we also quantified phospholipids, so that both natural sinks of FAs (phospholipids and TAGs) can be queried, as the reviewer suggested. These experiments show that storage into lipid droplets is increased in the ACC1 uORF mutant. We described these experiments in the text, as follows (lines 244-255):
"Since Acc1p activity is thought to be rate limiting for lipid biogenesis, we also examined if two major sinks of cellular lipids, neutral triglycerides (TAGs) in lipid droplets and phospholipids (PLs), were affected in the ACC1 uORF mutant. It was recently reported that storage of neutral lipids in lipid droplets fuels mitotic exit (Yang, Hsu et al., 2016). In rich medium with glucose as a carbon source, we observed that TAG levels increased somewhat in wild type cells arrested in mitosis

compared to exponentially proliferating cells (Figs. EV6A,C). In ACC1 uORF mutant cells, however, TAG levels were already high in exponentially growing cells and they did not increase further in mitosis (Figs. EV6B,C). On the other hand, total phospholipid pools were indistinguishable between wild type and ACC1 uORF mutant cells (Fig. EV6D). In exponentially proliferating cultures in poor medium, with glycerol as a carbon source, the ACC1 uORF mutant also had slightly higher levels of TAGs ($p<E-04$, Student's t test), but not phospholipids (Fig. EV7). Overall, these results argue that de-repressing the translational control of ACC1 increases the flux towards neutral lipid storage."

Can the authors explain how they think that changes in Acc1p activity (i.e. FA flux and more membrane phospholipids or more TAGs in lipid droplets?) lead to smaller cell size? Their discussion can be more concise once they obtain the lipid data from the uORF-Acc1 mutant.

RESPONSE: We now know that there are more TAGs in lipid droplets in the mutant. How this is related to the smaller cell size is not clear, and we have no explanation that we can state with confidence. Consequently, we prefer to simply state the finding (see line 311): "We noted that despite the increase storage of neutral lipids in the ACC1 uORF mutant (Fig. EV7), these cells are smaller than their wild type counterparts (Fig. 6)".

Other points:
I find that the timing of the Acc1p/Fas1p upregulation is not convincingly established; from the data in Figure 4, it is not clear to me when Acc1- and Fas-TAP protein levels peek. In Fig. 3B, the most significant increase takes place after 60 fL but the TAP-fusion sampling in Fig. 4 stop around 50fL, with a major increase between late 30s to 45 fL - which is not "late in the cell cycle" (l. 175).

RESPONSE: The reason is that diploid (larger) vs. haploid (smaller) cells are used in the different figures. As we had indicated in Table S2 and Methods, and now in the legend of Fig. 3, Fig 3 shows the results from diploid BY4743 cells. All other figures (e.g, Fig. 4 using epitope-tagged strains) show results in the haploid (BY4741) background. Furthermore, the position of the cell cycle in each experiment is shown by the percentage of budded cells in each figure. Hence, our statements "late in the cell cycle" are accurate and factual.

With respect to the experimental setup for sample collection (i.e. allowing each separate cell population to reach a set cell size before harvesting), I do not think the authors can talk about "periodicity" in Acc1p levels; that would require comparing samples for one division post-elutriation.

RESPONSE: As we had described in the text, the way the reviewer describes the required experiment is precisely how these experiments were done. In a time-series starting with early G1 cells, comparing sequential samples for one division post-elutriation, to monitor Acc1p, Fas1 and Fas2 -TAP levels in all the figures (e.g., Fig. 4). It was only in the ribosome profiling experiment that we had to generate cell-size series to collect enough cells for the experiment. This point is treated in detail in the text.

Referee #3:
This manuscript by H.M. Blank et al. investigates how the levels of individual proteins vary through the cell cycle of the yeast Saccharomyces cerevisiae. For a decade or two, numerous reports have appeared showing the variations in specific mRNAs at different cell-cycle stages, but these reports have contributed only marginally to understand the mechanisms coordinating cell growth and cell-cycle progression. In this manuscript, the translational activities of individual mRNAs as well as the levels of specific proteins at different points in the cell cycle have been measured. This, together with the use of centrifugal elutriation to obtain a population of synchronous cells that have not been exposed to much stress, makes this study important and significant.

In most previous reports using synchronously growing cells, stress has been employed to arrest the cells at a particular stage (temperature or drugs to arrest cell-cycle progression), and these methods have been shown to create artifacts and unwanted stress responses that perturb the cell

cycle. It is self-evident that in a culture in balanced growth, the cells at any particular stage in the cell cycle are, in principle, of the same size and have the same contents of macromolecules - otherwise the system is not in steady state. But the molecular mechanisms governing this steady state are not known. In this work the authors show that towards the end of a minimally perturbed cell cycle, in late G2, mRNAs related to lipogenesis are preferentially translated, maybe giving the first clues to the elusive coupling mechanism. These data were accumulated by doing ribosome profiling of the RNA extracted from cells emanating synchronously from the unbudded state in early G1 phase.
Although the method of centrifugal elutriation has the great advantage of producing cells that are little affected by the treatment itself, as opposed to other methods creating synchronous populations, it has the drawback of giving a lower degree of synchrony. This can be exemplified by Figure 1C, where the level of certain mRNAs' attachment to ribosomes is plotted as a function of cell size. It is not absolutely clear, but it seems that the 2- to 3-fold increase observed for CLN1 and CLN2 appear over a fairly large size range (from 45 to 55 fL).

The uncertainty is manifested partly because of another problem, namely that high numbers of cells are required to perform the ribosome profiling experiment, and enough cells cannot be collected to perform a time series of one particular culture. Therefore, parallel samples were used and the assumption is that they are all similar. The alternative is to become "greedy" when same-size cells are collected, resulting in a wider variability is cell sizes and therefore lowered synchrony. It seems that the reviewers have made wise decisions and trade-offs to create data that can be interpreted with confidence. An indication of the degree of synchrony, such as a measurement of the kinetics of S phase entry and progression by flow cytometry, might be helpful.

RESPONSE: The synchrony achieved by our elutriation protocol is exceptional. The y-axis scale in Fig. 1C is in $\log_2$, hence the actual increase is >20-fold in CLN1, CLN2 transcript levels, exactly as expected. These molecular markers, together with an independent cytological metric (budding), and the concordance with the published data about the behavior (i.e., critical size) of this strain in the same media (see the Hoose et al citation) fully support and justify our conclusions.

The approach is novel and the results are interesting. The conclusions are well supported by the data presented. The manuscript is well written and relatively easy to understand.
Some specific comments, in the order of appearance in the manuscript:
1. Line 1. I am not sure why "growing" should be in the title. Maybe even "synchronous" should be deleted? "Translational control of lipogenic enzymes in the cell cycle of yeast cells" is perfectly OK.

RESPONSE: We appreciate the suggestion but the “growing, cycling” words underscore the distinction with all other studies that examined translational control in the cell cycle.

2. Line 39. The statement that cell growth depends on protein synthesis appears rather obvious; I am not sure which part of Mitchison's work is referred to.

RESPONSE: As we cite in the text (Cell Growth and Protein Synthesis. The Biology of the Cell Cycle", Cambridge University Press; pp 129., 1971), the statement is: ““No sensible interpretation of cell growth can be made without a knowledge of the overall pattern of protein synthesis”.

3. Line 43. May link up with the previous: It is pretty obvious that larger cells produce more proteins than small cells. And it does not follow from the statement before, that cell size is increasing exponentially through the cell cycle. Some stringency is required for these statements to become meaningful.

RESPONSE: It is not clear to us what point the reviewer is making here.

4. Line 105. An indication of how good the synchrony is, would be good to have. This will add confidence to the data and help their interpretation. It is possible that the synchrony is very good, which might suggest that an improvement in method might give better data and sharper changes in gene expression.

RESPONSE: Please see our response to the first comment this reviewer made.

5. Line 225. It is said that all cell-cycle phases are increased in length, but they have not been measured. What is shown is that G1 and G2/M are increased, but nothing is known about S phase or G2 and M individually. Should be modified.

RESPONSE: We modified the text as suggested. It now reads "…an increase in the duration of the G1 and G2/M cell cycle phases."

6. Line 260. There will probably always be some kind of coupling between growth and cell division. The point here is that the NORMAL coupling that occurs in unperturbed cultures is likely to be maintained.

RESPONSE: We modified the text as suggested.

7. Line 266. This line is a bit cryptic; the contents should be spelled out more clearly.
RESPONSE: The sentence now reads as follows: "…the data indicate that that the translational efficiency of *ACC1* is regulated in the cell cycle and also in response to nutrient availability."

8. Line 296. It is not obvious that the Yao et al paper is relevant. Reducing the access of any nutrient to a bacterial population will undoubtedly reduce the cell size. But it does not necessarily imply that this nutrient is normally regulating cell size.

RESPONSE: We deleted the sentence.

9. Line 367. It would be helpful to indicate which chamber was used. This centrifuge does have rather large chambers, and it would be good to know that the largest one was used. Otherwise, the method can be improved.

RESPONSE: Yes, we used the large chamber in all experiments, and added this information (lines 383): "the culture was then loaded at a pump speed of 50 ml/min onto a large elutriator chamber (40 mL) spinning at 3200 rpm".

10. Figure 6. If this is a culture growing in steady state the growth rates given in panel A should correspond to the specific growth rates plotted in panel C, bottom. The exact values are not clear, but it does not seem that the numbers correspond very well. This should be addressed.

RESPONSE: The numbers do not and should not match exactly because in panel C we are only looking at daughter cells, which are born much smaller than their mothers, hence the cell cycle of daughters is significantly longer than the cell cycle of their mothers, while in panel A we are looking at mixed asynchronous populations, mothers and daughters. Furthermore, in C we measure rate of cell size increase, while in A rates of cell division.
Furthermore, the p values are given above the line connecting the data sets for the two strains, as if the "distance" between the strains is the subject. If it is the difference in the ordinate values that is the matter, the notation should be changed.

RESPONSE: We changed the notation, as suggested.

Referee #4:
Regarding the request to evaluate the technical competency of this paper, I think the authors have made an effort to characterize the changes in translation with growth. However, one caveat of their approach is that they have used polyA+ to quantify mRNA levels in order to normalize the ribosome footprints and calculate translation efficiency. However, how do the authors know that changes in translation efficiency are not instead increase or decrease in the polyA+ mRNA population rather than changes in translation per se?

RESPONSE: The reviewer points to the common problem of "spurious correlation" encountered in some of these studies. Although we had not explicitly mentioned it in the paper, one of the statistical packages we used to analyze our data (*anota*) was specifically developed to tackle this problem. We added the following in the text (line 504) to address this point. "Note that the anota package incorporates analysis of partial variance, which eliminates spurious correlations arising from the

possibility that translational efficiency scores may correlate with cytoplasmic mRNA abundance instead of true translational efficiency (Larsson et al., 2011)."

2nd Editorial Decision 01 November 2016

Dear Dr. Polymenis,

Thank you for submitting a revised version of your manuscript. It has now been seen by two of the original referees and their comments are shown below. As you will see they both find that all criticisms have been sufficiently addressed (apart from a minor clarification from ref #2) and recommend the manuscript for publication. However, before we can go on to officially accept your manuscript there are a few editorial issues concerning text and figures that I need you to address in a final revision:

-> We can accommodate up to 5 Expanded View figures per paper in The EMBO Journal and I noticed that your manuscript currently has 8. I would therefore ask you to either combine some of these figures to yield a total of 5 or move 3 of them to the Appendix file. Please also update the figure legends and call-outs in the text accordingly.

-> For the provided datasets, these should only be labeled as source data when depicting raw data underlying specific figures (and in that case be labeled as source data for the respective figure). Otherwise, they can be included as EV tables. Please confer with our authors guidelines online and feel free contact us with any specific formatting questions.

-> Please also make sure that all EV figures and data sets are mentioned in the main text (currently missing for fig EV8 and dataset 4)

-> Please add scale bars in the cell images in figures EV6 and EV7 and include the length of the scale bar in the figure legends.

-> We noticed that the Western blots in several cases appear rather strongly contrasted. I would encourage you to use the original scans of blots whenever possible.

-> We generally encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to your final revision.

REFEREE REPORTS

Referee #2:

The authors addressed my main criticism. One last correction is required: the authors quantify fluorescence of lipid droplets (neutral lipids) and not TAGs - the text (lines 249, 253, 1016, 1025) must be corrected.

Referee #3:

The ms is now submitted for a second time. The comments and criticism from the reviewers have

been taken care of in a good way and the manuscript is now better. Not all of my minor comments were addressed appropriately, but that is not important and undoubtedly a matter of debate. It is my opinion that this manuscript is now very good.

2nd Revision - authors' response 09 November 2016

-> We can accommodate up to 5 Expanded View figures per paper in The EMBO Journal and I noticed that your manuscript currently has 8. I would therefore ask you to either combine some of these figures to yield a total of 5 or move 3 of them to the Appendix file. Please also update the figure legends and call-outs in the text accordingly.

RESPONSE: Completed as requested. We moved three EV Figures to the Appendix.

-> For the provided datasets, these should only be labeled as source data when depicting raw data underlying specific figures (and in that case be labeled as source data for the respective figure). Otherwise, they can be included as EV tables. Please confer with our authors guidelines online and feel free contact us with any specific formatting questions.

RESPONSE: We have now uploaded the Datasets as Source Data for the corresponding figures. We used a compressed .zip format, to avoid their conversion to pdf files (as suggested by the EMBO Office) and maintaining their utility as .txt files.

-> Please also make sure that all EV figures and data sets are mentioned in the main text (currently missing for fig EV8 and dataset 4)

RESPONSE: Corrected as requested.

-> Please add scale bars in the cell images in figures EV6 and EV7 and include the length of the scale bar in the figure legends.

RESPONSE: Corrected as requested. These Figures are now EV3 and EV4.

-> We noticed that the Western blots in several cases appear rather strongly contrasted. I would encourage you to use the original scans of blots whenever possible.

RESPONSE: Corrected as requested.

-> We generally encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

RESPONSE: Completed as requested.

REFEREE #2:
The authors addressed my main criticism. One last correction is required: the authors quantify fluorescence of lipid droplets (neutral lipids) and not TAGs - the text (lines 249, 253, 1016, 1025) must be corrected.

RESPONSE: Corrected as requested, not only in the text, but also in the figures.

3rd Editorial Decision 18 November 2016

Thank you for submitting the final revision of your manuscript to The EMBO Journal. I am happy to let you know that you study has now been officially accepted for publication here

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Polymenis, Michael
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2016-95050R

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs 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 and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

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.

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

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical methods were used to predetermine sample size.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Not applicable.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Not applicable.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	The experiments were not randomized.
For animal studies, include a statement about randomization even if no randomization was used.	Not applicable.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	The investigators were not blinded to allocation during experiments and outcome assessment.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Not applicable.
5. For every figure, are statistical tests justified as appropriate?	Data in Figures 1,4,5,6 7,EV1,EV2,EV5 are shown descriptively, with no statistics. For analysis of the sequncing data (Figures 2 and 3) for ribosome and transcriptome profiling, specialized statistical packages were used (cycle, anota, babel), as described in the text and the cited literature. For the the data in Figures EV3,4 to assess the significance of differences among cultures and conditions, non-parametric, distribution-free tests were used. In every case the sample size is shown.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We used non-parametric, distribution-free tests: Wilcoxon rank sum test for two-sample comparisons and for pairwise multiple comparisons the Wallis and Kruskal one-way analysis of variance by ranks, followed by the post-hoc Nemenyi test.
Is there an estimate of variation within each group of data?	In each case with n<5, each data point is shown individually, to reflect the variation of the data. For the rest of the data (Figures EV3,4) they were shown as box-plots.
Is the variance similar between the groups that are being statistically compared?	The variance of the groups compared was similar and displayed in srtripcharts and box-plots, as indicated.

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C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	http://dx.doi.org/10.1006/meth.2001.1183
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Not applicable.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Not applicable.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Not applicable.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Not applicable.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not applicable.
12. 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.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not applicable.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not applicable.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not applicable.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not applicable.
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not applicable.

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	GEO: GSE81932
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	All datasets have been deposited at the above accession code.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	Not applicable.
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	DATA AVAILABILITY All the primary sequencing data and datasets reported in this paper are available from the Gene Expression Omnibus (accession number GSE81932).
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	Not applicable.

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No biosecurity, dual use research is reported.