Cell cycle-independent integration of stress signals by Xbp1 promotes Non-G1/G0 quiescence entry

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Transaction Report:

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Re: JCB manuscript #202103171

Dr. Orlando Arguello-Miranda
The University of Texas Southwestern Medical Center
Lyda Hill department of Bioinformatics
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Dallas, Texas 75390

Dear Dr. Arguello-Miranda,

Thank you for submitting your manuscript entitled "Cell cycle-independent integration of stress signals promotes Non-G1/G0 quiescence entry". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

As you will see, the reviewers were overall enthusiastic about your work, but they raised a number of substantial concerns that will need to be addressed before the paper would be deemed appropriate for publication in JCB. The reviewers note that the study offers insufficient direct evidence to support that Xbp1 drives entry of cells into non-G1 (high-Cdk1) quiescence. They also ask for further insights into the nature and breadth of this phenomenon to more physiological insults. We hope that you will be able to address these concerns with substantive new data, as well as that you will be able to tackle the rest of the referees' points.

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

GENERAL GUIDELINES:

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

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

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

Supplemental information: There are strict limits on the allowable amount of supplemental data.

Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

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

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

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

Sincerely,

Tobias Meyer
Monitoring Editor
Journal of Cell Biology

Lucia Morgado Palacin, PhD
Scientific Editor
Journal of Cell Biology

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

Argüello-Miranda et al. present results of microfluidics studies of yeast cells responding to starvation. They show that a portion of the cells can enter to a quiescent state from high Cdk activity G2-M state and this ratio can be increased by pre-stressing the cells. They found nuclear Xbp1 level as a good predictor of possible high-Cdk1 quiescence entry and perturbations in Xbp1 level can influence this entry point. They also suggest that Xbp1 is a time-delayed integrator of stress history of cells.

The manuscript is nicely written and contains a great combination of advanced multicolour imaging with machine learning algorithms to reach interesting biological conclusions. The concept that cells (also yeast cells) can enter into quiescence from G2 is not new, but the molecular mechanisms affecting this are unknown. The finding that Xbp1 is a key controller is relevant and it explains how stress pathways affect quiescence entry, which is a significant result worth to be published in JCB. Although it is still not clear how Xbp1 induced transcriptional changes block the cells in a high-Cdk

state. The manuscript could be published in JCB after a few minor modifications and clarifications:

- Although the authors claim that "Single-cell tracking of budded and unbudded quiescent cells showed no significant differences in cell volume (Fig. 1 J)", this figure and Fig S1A also show that budded cells are a bit smaller than unbudded. Is there any explanation on this?

- On Fig 3A it would be useful to label the cells, which were selected as high-Cdk by the algorithm.

- Fig 5 G and I shows that Xbp1 only slowly increasing upon starvation stresses, while on Fig 8 E and F the same Xbp1 nuclear intensity seem to increase much faster (timescales differ, so unclear how large is the difference). What could cause this difference?

- Fig 6D representative example shows a really good posterior, but the average of many of such runs on 6H shows a much reduced value with the data on 6D out of the shown distribution. Maybe a better, more representative example should be used on 6D, or this difference should be explained.

- On fig 8A the half-life of Xbp1 transferred to rich medium is approx. 3 hours, 8H also shows a similar value after 12h starvation, while 8K shows almost 8 hours half-life. What causes this difference?

- Can the authors comment on which of the many targets of Xbp1 might be relevant for the observed high-Cdk1 block?

- The experiments were performed on diploid W303 strains. How far the results could be specific for this strain, and how far this is specific for diploid cells?

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

This is an interesting and well-executed manuscript in which the authors carefully investigate the response of yeast to nutrient starvation. The authors make the interesting and surprising discovery that there are different responses, including yeast that form a bud and remain in that state, with a small bud that doesn't grow or disappear, a state the authors characterize as "high CDK1." While the authors argue that high CDK1 quiescent states exist in other contexts, they report that it has been difficult to study and characterize high CDK1 states in complex tissues. As a result, the existence of such a state and whether it reflects a failure to arrest properly, slowly dividing cells, or a regulated biological response is controversial.

The authors tackle this problem in starvation-induced quiescence in Saccharomyces cerevisiae using real time imaging, microfluidics and machine learning algorithms. They learn that these cells that form buds and remain in that state have similar changes to low CDK1 quiescent cells such as changes in autophagy, protein aggregation and increase in mitochondria. Yet, the low CDK1 quiescent cells exhibit nuclear accumulation of transcription factors Xbp1, Gln3 and Sfp1. In particular, the authors highlight Xbp1 as a transcription factor that accumulates in low CDK1 quiescent cells and that, when eliminated, results in fewer CDK1 quiescent cells. They further show that levels of nuclear Xbp1 accumulate with stress, thus providing a way for the cell to integrate information about previous stress signals.

Their work carefully distinguishing among the different responses of individual yeast cells to

starvation will be valuable for the field of quiescence and cell cycle research. The focus on the ER stress response gene Xbp1 may provide a new molecular focus for understanding how cells integrate information to determine whether to proliferate or quiescence and if they quiesce, in what manner.

Comments

The relevance of the manuscript's findings about a non-G1 quiescent state are somewhat limited by the use of severe extreme starvation. This signal may be relevant for yeast, which may face extreme starvation in the wild, but the relationship to quiescence in multi-cellular organisms, which would rarely face true starvation of all nutrients, is unclear. There are references to high CDK1 arrest in multicellular organisms in the introduction, but is this the same state?

The high CDK1 quiescent cells arrested with a small bud and didn't progress. It's not clear what state the cells are in or why they are stopping. Are the cells depleted of ATP? Are their energy stores too low to continue budding? Are they devoid of a key protein or other factor required to continue?

Sic1 levels are low in the high CDK1 quiescent cells, consistent with high CDK1 activity. It would be helpful to also characterize Far1 so that the behavior of all possible CKIs in established.

By only using a complete starvation arrest for the first few figures, it is particularly difficult to understand what the high CDK1 quiescent state is because it is not clear whether it would occur from a less extreme limitation. Providing information on additional arrest signals earlier in the manuscript could shed light on the nature of the state and how it relates to more traditional quiescence.

XBP1 levels are associated with high G1 CDK quiescence and XBP1 inactivation results in fewer quiescent cells. The authors show that the viability of the quiescent cells is much lower when XBP1 is inactivated. Is it possible that expressing Xbp1 allowed the high Cdk1 quiescent cells to survive rather than inducing quiescence?

Is it possible that another gene induces the high CDK1 quiescence cell cycle arrest, and without XBP1, the quiescent cells die, so XBP1 is a survival factor rather than a quiescence regulator?

In Fig 8J, it looks like Stb3 levels are higher in the 2nd quiescence than the first, with a difference similar to that observed for Xbp1. This would seem to undermine the argument that Xbp1 alone is the crucial determinant of the cell's fate.

Reviewer #3 (Comments to the Authors (Required)):

In this paper by Arguello-Miranda et al, the authors show that acute starvation induce the emergence of 3 types of cells : direct G1, post-mitotic G1, and Non-G1 that respectively arrest in G1, finish their ongoing cell cycle and arrest in G1, or cease dividing as non-G1 cells. While the 2 first cell categories display a low Cdk1 activity, the latter has a high-Cdk1 activity as attested by several markers (persistent Cdc10 ring, no nuclear accumulation of Sic1 etc…), the cell types having the same survival rate. Then they show that high-Cdk1 Q-cells display an increased nuclear accumulation of Xbp1, Gln3, and Sfp1. They further show that high-Cdk1 quiescence was not

defined by cell cycle stage at starvation onset but was rather determined by the stress status of the cell. They convincingly demonstrate that Xbp1 was a causative factor of high-Cdk1 quiescence entry as Xbp1 is sufficient to increase the frequency of high-Cdk1 Q-cells when accumulated at high levels (this effect requiring Xbp1 binding to DNA) and as the deletion of XBP1 reduced the total fraction and viability of high-Cdk1 quiescent cells. Finally, they show that Xbp1 may acted as an integrator of previous stress stimuli, which rendered the propensity of high-Cdk1 quiescence dependent on individual-cell history.

This study is of great interest for the cell biologist community and overall, the data are very convincing.

Major

1-Quiescent cells arrested in other cell cycle phases than G1 have been previously described in budding yeast by the Broek lab (Wei et al, 1993) and the Sagot lab (Laporte et al, JCB 2011 and eLife 2018). It was shown that these cells are arrested in the cell cycle as non-G1 cells (budded cells) and that individually, these cells are able to resume proliferation. The results presented here upon acute starvation confirm these previous findings in batch and clearly push them forward. Notwithstanding, the authors should clearly cite these previous studies in the introduction and further discussed them in the discussion section. In fact, it would be interesting to compare the results previously published upon slow carbon exhaustion in YPD batch and pads and the results obtained here upon acute starvation of both carbon and nitrogen. To unable this comparison, the authors should clearly show the percentage of direct G1, post-mitotic G1, and non-G1 cells in the total population. Within the high-Cdk1/Non-G1 cells, they should also give the percentage of cells in S-phase (using the Cdc7 experiment of Fig2I) and the percentage of short spindle and anaphase B cells (using the Spc42 data of Fig2H). In the same vein, it should be interesting to know what king of cells undergo the one last division (the post-mitotic G1). It is all kind of cells or just the big mother cells that have already passed the critical size and the start point in G1?

2- After Fig2, the authors focus on post-mitotic G1 (low-Cdk1 quiescence) and Non-G1 (high-Cdk1 quiescence). Why they do not show their results for direct G1? It would be very interesting to see what is the localization of the markers of Fig3 and Fig4 in these cells. Are direct G1 displaying autophagosomes? Normal mitochondria? Q/N aggregates? Rad52 foci? Etc... Of note, Laporte et al (JCB 2011), have shown that actin bodies, proteasome granules and mitochondria reorganized identically in both budded and unbudded quiescent cells. This should be mentioned when describing Fig4.

Furthermore, the images shown in Fig3 and Fig4 are of very poor quality. Probably because the authors utilized a 40X 1.3 NA objective. Can the author use a better objective? Would a 63X 1.4NA be compatible with the Cell Asics setting? If not, the authors should present images to convince the reader about of the observed changes in localization. For examples, in Fig 3, I do not see any Sfp1 cytoplasmic clusters appearing, etc...; in Fig4, mitochondria look like clumps (actually, the authors should explain how did they measure what they call mitochondria "biomass"), I do not see any Rad52 foci but rather a blurry fluorescent nucleus.... Showing better quality images and statistics for the 3 cell categories (direct G1, post-mitotic G1, and non-G1) would greatly improve the data.

3- In Xbp1 delta cells, it is of critical interest to give the % of each cell category (direct G1, post-mitotic G1, and non-G1) and their respective viability. Fig7I show that there is very little high-Cdk1 cells in a Xbp1 delta population and that these cells massively die upon starvation. What is the viability of the Xbp1 delta low-cdkA cells? This is critical to better comprehend the function on Xbp1 in quiescence establishment.

4- They author claim that Xbp1 acted as an integrator of previous stress stimuli, which rendered the propensity of high-Cdk1 quiescence dependent on single-cell history. Although very appealing, the text should be soften given the fact that Xbp1 "history" is lost after 4h in rich medium (Fig. 8H).

In fact, the cumulative effect observed in Fig8 E and F is due to the short period of nutrient replenishment between two starvations. Cells have hardly the time to re-enter the division cycle and divide once….so given Fig 8B, data obtained in 8E and F are trivial. Fig8K is more convincing, but again 8h hours of nutrient replenishment may hardly allows 4 divisions, not enough to dilute Xbp1. In the text the conclusions have to be soften accordingly.

Importantly, given that high-Cdk1 cells hardly represent 10% of the total cell population, the authors should add the proportion of each cell types in a WT population in the model presented in Fig8N (90% next to low cdk, 10% next to high cdk).

Minor :
1- The use of the term G0 in the text and Fig8N is not appropriate, especially if cells are budding.
2- In the last sentence of the introduction: "Our results show that cell cycle independent integration of stress stimuli by transcriptional repressors is a viable cellular strategy to establish quiescence outside of a G1/G0 state", the authors utilize the term "strategy".
In this study, it just happen that Xbp1 accumulates upon repetitive acute starvation - so OK for the term "integrator" of past stress signals. Yet, the "integration" is rapidly lost by dilution after a few divisions… The consequent arrest as non-G1 cells can not be defined as a STRATEGY. At this step, it is just an observation, not a specific cell "program". Thus this specific part of the text has to be soften to better fit with the conclusions from the experiments.

Revision of manuscript:
"Cell cycle-independent integration of stress signals promotes Non-G1/G0 quiescence entry"

We thank the reviewers for their insightful comments. We have revised the manuscript to address all points raised by the reviewers. This included performing 3 new experiments, generating 6 new strains, quantifying data from 8 pre-existing experiments, and re-writing 11 sentences in the original manuscript. The new experiments are accommodated in supplementary material, and one key new experiment is included in Fig. 8 N. A new version of Video 6 with improved labeling was added. Re-written sentences appear highlighted in yellow in the revised version of the manuscript.
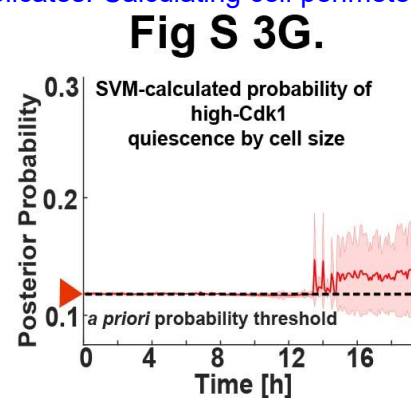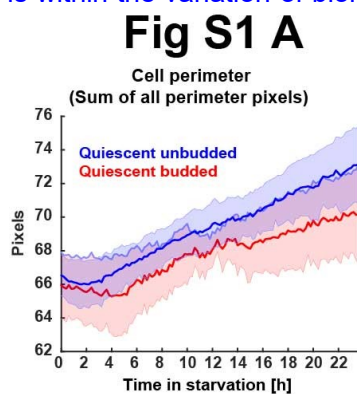
Here you will find the original reviewers' comments in bold black and our point-by-point replies in blue. Whenever changes to figures or new quantifications were required, we included them in our response to ease comparisons.

Point-by-point response to reviewer's comments:

Reviewer #1: *Argüello-Miranda et al. present results of microfluidics studies of yeast cells responding to starvation. They show that a portion of the cells can enter to a quiescent state from high Cdk activity G2-M state and this ratio can be increased by pre-stressing the cells. They found nuclear Xbp1 level as a good predictor of possible high-Cdk1 quiescence entry and perturbations in Xbp1 level can influence this entry point. They also suggest that Xbp1 is a time-delayed integrator of stress history of cells. The manuscript is nicely written and contains a great combination of advanced multicolour imaging with machine learning algorithms to reach interesting biological conclusions. The concept that cells (also yeast cells) can enter into quiescence from G2 is not new, but the molecular mechanisms affecting this are unknown. The finding that Xbp1 is a key controller is relevant and it explains how stress pathways affect quiescence entry, which is a significant result worth to be published in JCB. Although it is still not clear how Xbp1 induced transcriptional changes block the cells in a high-Cdk state. The manuscript could be published in JCB after a few minor modifications and clarifications:*

- Although the authors claim that "Single-cell tracking of budded and unbudded quiescent cells showed no significant differences in cell volume (Fig. 1 J)", this figure and Fig S1A also show that budded cells are a bit smaller than unbudded. Is there any explanation on this?

Thank you for bringing this to our attention. Fig. 1 J shows no significant difference in the size time series for budded and unbudded cells as judged by the overlap in the 95% confidence interval. In other words, the difference is within the variation of biological replicates. Calculating cell perimeter, or the predictive power of size for High-Cdk1 Q-cells, confirms this result. We now show cell perimeter in Fig S1 A and include the Support Vector Machine (SVM) predictive power analysis for cell size in Fig S3 G (shown here on the right):

Fig S1 A
Cell perimeter
(Sum of all perimeter pixels)
Quiescent unbudded
Quiescent budded

Fig S 3G.
SVM-calculated probability of high-Cdk1 quiescence by cell size
a priori probability threshold

- On Fig 3A it would be useful to label the cells, which were selected as high-Cdk by the algorithm.

The High Cdk1 Q-cell in the representative phase-contrast micrograph of Fig 3 A is now labeled with a yellow arrow (shown here on the right):
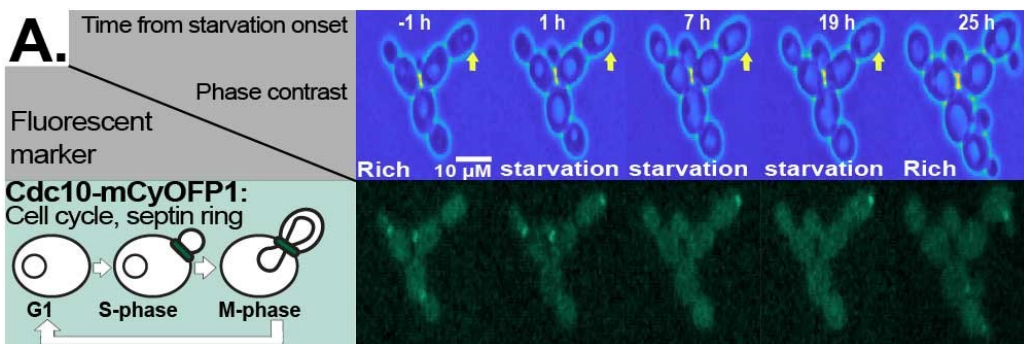

- Fig 5 G and I shows that Xbp1 only slowly increasing upon starvation stresses, while on Fig 8 E and F the same Xbp1 nuclear intensity seem to increase much faster (timescales differ, so unclear how large is the difference). What could cause this difference?

The perceived difference is due to different experimental conditions:
-Fig 8, E & F show cells exposed to a single starvation event, from rich to starvation medium.
-Fig 5, G & I show cells exposed to repeated starvation events between glucose/nitrogen depleted medium and rich medium. As expected from a time-delayed signal integrator, Xbp1 increases faster after repeated starvation events.

The only time window in which Fig 8, E & F, and Fig 5, G & I are comparable is the first hour after transfer to poor medium. Plotting the first hour of these experiments using the same scale in the X-axis (time, min) and the Y-axis (Xbp1 nuclear intensity scaled between 0-100) shows a consistent and similar Xbp1 behavior (shown here below):
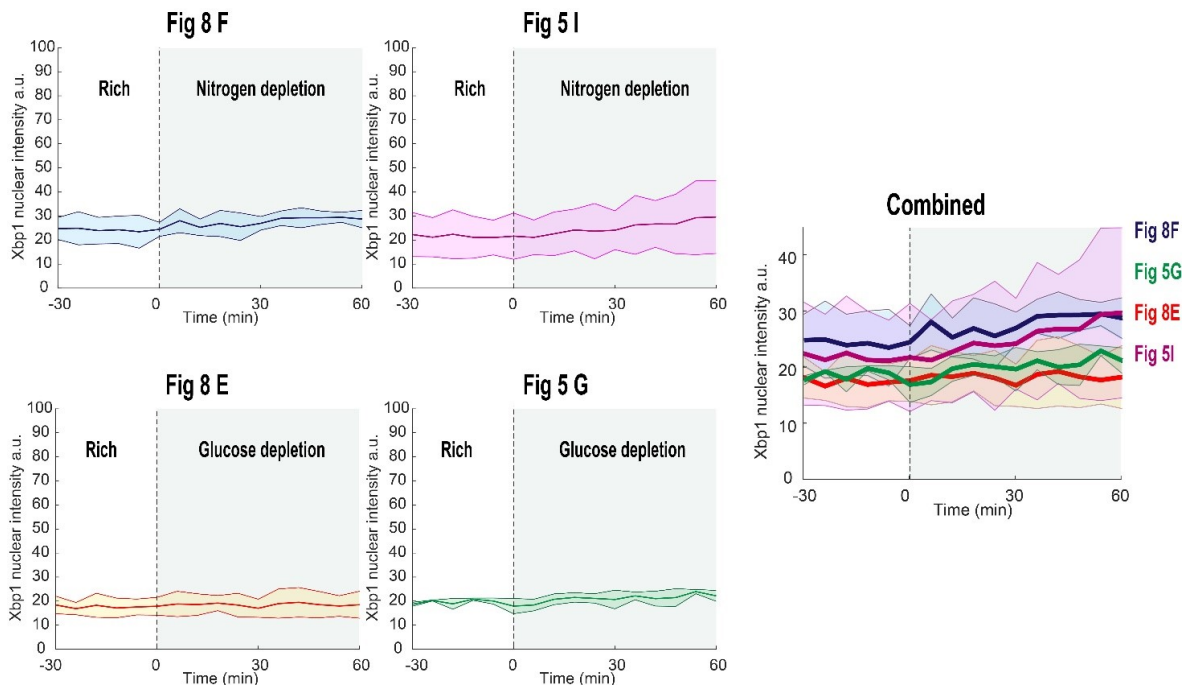
- Fig 6D representative example shows a really good posterior, but the average of many of such runs on 6H shows a much reduced value with the data on 6D out of the shown distribution. Maybe a better, more representative example should be used on 6D, or this difference should be explained.

We agree with this point. The time series for cell no. 568 previously displayed in Fig 6 D has been replaced by a more representative example using cell no. 769:
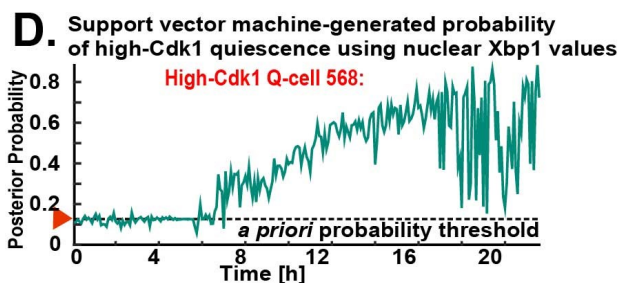
Old Fig 6D   New Fig 6D
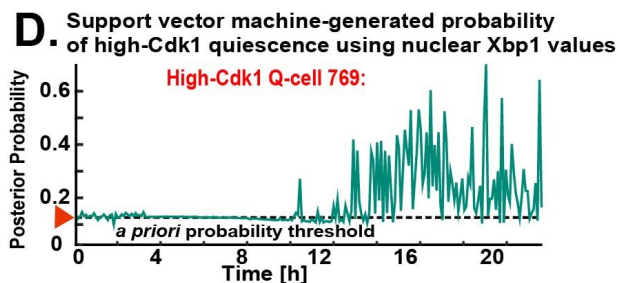
- On fig 8A the half-life of Xbp1 transferred to rich medium is approx. 3 hours, 8H also shows a similar value after 12h starvation, while 8K shows almost 8 hours half-life. What causes this difference?

The perceived difference is caused by differences in the Y axis:

-Fig 8A shows multiple stress transcription factors during a 12 h period centered around t0 = return to rich medium. The Y-axis (Nuclear intensity) is scaled so that 100 corresponds to the highest nuclear level during the 12h period. The scaling is necessary to quickly visualize all different transcription factors in the same plot.

- the Y-axis of Fig 8K is not scaled because the emphasis is on comparing the values of a single protein, Xbp1, during the first and second quiescence entry.

Plotting the two Xbp1 data sets using the same scaled Y-axis during a 12 h period centered around t0 = return to rich medium shows that there's no significant difference in the decay of Xbp1 in those two experiments during the first 4 h upon return to rich medium (here on the right). We now
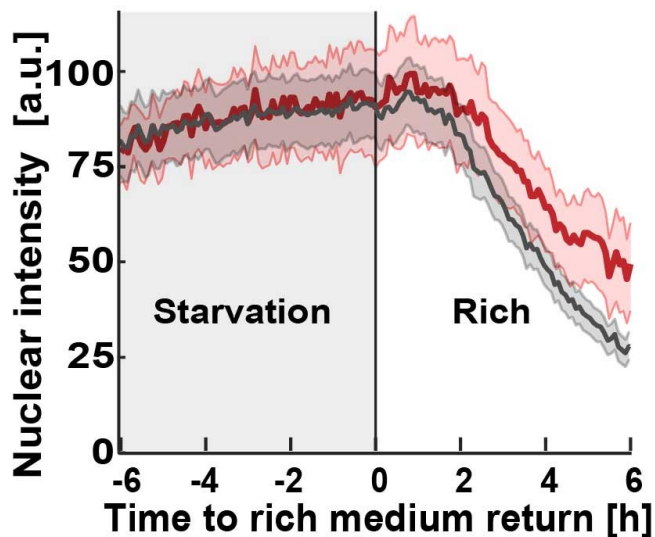
expanded the description of the scaling process in Fig 8A's legend:

"(A) Average nuclear intensity of Sfp1-mScarlet-I, Stb3-mTFP1 and Xbp1-mNG, as percentage of their maximum nuclear accumulation ± 6h around transfer to rich medium"

- Can the authors comment on which of the many targets of Xbp1 might be relevant for the observed high-Cdk1 block?

We have now addressed this. We have expanded the list of relevant Xbp1 targets mentioned in the discussion:

"Although Xbp1 represses crucial cell cycle genes involved in proliferation-promoting positive feedback loops, such as cyclins (CLN3, CLB2), mitotic regulators (CDC5, CDC20) and mitotic transcriptional activators (NDD1) (Miles et al., 2013), it has been considered… ".

We also stated in the discussion that these genes engage in proliferation-promoting positive feedback loops:

"We envision that, under persistent starvation, high levels of Xbp1 force cells to settle in the available stable-steady state by inhibiting genes required for proliferation-promoting positive feedback loops through transcriptional repression."
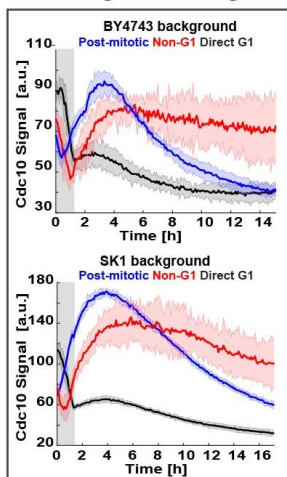
- The experiments were performed on diploid W303 strains. How far the results could be specific for this strain, and how far this is specific for diploid cells?

We now show quiescence entry experiments in two different *S. cerevisiae* strains, BY4743 and SK1, and in haploid or diploid strains. High-Cdk1 quiescent cells are produced upon acute starvation regardless of strain genetic background or ploidy. We have included this information as S Fig. 1 E, H & F (shown here below) and included the following sentence in the results section:

"Cdc10-cluster classification was independent of ploidy or strain genetic background (Fig. S 1 E, F & H)"
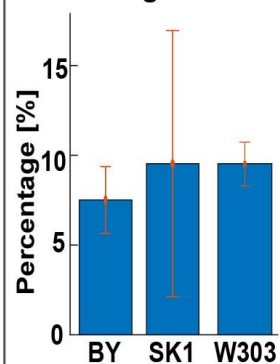

Fig S1 E

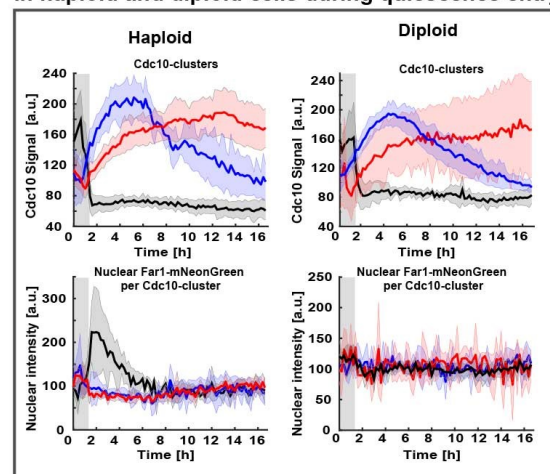Cdc10-clusters in *S. cerevisiae* BY or SK1 genetic backgrounds

Fig S1 H

Fig S1 F

Far1-mNeonGreen nuclear levels per Cdc10 cluster in haploid and diploid cells during quiescence entry

Reviewer #2: *This is an interesting and well-executed manuscript in which the authors carefully investigate the response of yeast to nutrient starvation. The authors make the interesting and surprising discovery that there are different responses, including yeast that form a bud and remain in that state, with a small bud that doesn't grow or disappear, a state the authors characterize as "high CDK1." While the authors argue that high CDK1 quiescent states exist in other contexts, they report that it has been difficult to study and characterize high CDK1 states in complex tissues. As a result, the existence of such a state and whether it reflects a failure to arrest properly, slowly dividing cells, or a regulated biological response is controversial. The authors tackle this problem in starvation-induced quiescence in Saccharomyces cerevisiae using real time imaging, microfluidics and machine learning algorithms. They learn that these cells that form buds and remain in that state have similar changes to low CDK1 quiescent cells such as changes in autophagy, protein aggregation and increase in mitochondria. Yet, the low CDK1 quiescent cells exhibit nuclear accumulation of transcription factors Xbp1, Gln3 and Sfp1. In particular, the authors highlight Xbp1 as a transcription factor that accumulates in low CDK1 quiescent cells and that, when eliminated, results in fewer CDK1 quiescent cells. They further show that levels of nuclear Xbp1 accumulate with stress, thus providing a way for the cell to integrate information about previous stress signals. Their work carefully distinguishing among the different responses of individual yeast cells to starvation will be valuable for the field of quiescence and cell cycle research. The focus on the ER stress response gene Xbp1 may provide a new molecular focus for understanding how cells integrate information to determine whether to proliferate or quiescence and if they quiesce, in what manner.*

Reviewer 2 point 1: The relevance of the manuscript's findings about a non-G1 quiescent state are somewhat limited by the use of severe extreme starvation. This signal may be relevant for yeast, which may face extreme starvation in the wild, but the relationship to quiescence in multi-cellular organisms, which would rarely face true starvation of all nutrients, is unclear. There are references to high CDK1 arrest in multicellular organisms in the introduction, but is this the same state?

We address this point as 2 questions:

A) "relevance of the manuscript's findings about a non-G1 quiescent state are somewhat limited by the use of severe extreme starvation."

The acute starvation stimulus used in this work to trigger quiescence has been extensively used in other fields of yeast research to provide molecular mechanisms for processes such as meiosis and stress responses. For instance, acutely starved sporulating yeast cells are a powerful model for meiosis. Key enzymes for reductional chromosome segregation (such as Mam1, discovered by Attila Toth while in Kim Nasmyth's group) or meiotic DNA recombination (such as Spo11, discovered by Scott Keeney while in Nancy Hollingsworth's group) were first described in acutely starved yeasts cells. The fact that mammalian cells enter meiosis using a carefully orchestrated developmental program while yeasts use acute starvation has not prevented using yeast as a meiosis model because the crucial meiotic processes (two rounds of chromosome segregation without intervening DNA replication) are a fundamental modification of a conserved cell cycle machinery. In a similar manner, we hope that our work provides an example of how the crucial problem of cellular quiescence entry (how to halt proliferation under stress safely) can be addressed using acutely starved yeast cells under microfluidics conditions.

B) There are references to high CDK1 arrest in multicellular organisms in the introduction, but is this the same state?

Using Cdk1 activity as a classification criterion, the quiescent states described in the introduction are comparable to the high-Cdk1 state we describe in this paper. Using cell cycle stage as a classification criterion, our High-Cdk1 cells resemble G2 quiescent cells because they replicate DNA and separate spindle pole bodies, producing a phenotype similar to the High-Cdk1 quiescent cells observed in *D. melanogaster*'s neural progenitors (Otsuki and Brand, 2018). Using the requirement of histone deacetylase regulators as a criterion, High-Cdk1 Q-cells are like quiescent mouse oocytes because both depend on histone deacetylase regulators such as Xbp1 or HDAC3 (Wang et al., 2019). In conclusion, different comparisons could be made, but previous studies lack the single-cell resolution required to make a final assessment. Therefore, in this manuscript, the most conservative classification according to Cdk1-activity is made.

Reviewer 2 point 2: The high CDK1 quiescent cells arrested with a small bud and didn't progress. It's not clear what state the cells are in or why they are stopping. Are the cells depleted of ATP? Are their energy stores too low to continue budding? Are they devoid of a key protein or other factor required to continue?

Several lines of evidence show that High-Cdk1 quiescent cells have fully functional biosynthetic, nuclear import/export, energy storage, and ATP producing machinery:

1- Functional protein synthesis machinery: High-Cdk1 cells can produce proteins under the control of CUP1 or MET3 inducible promoters (such as N-terminal Ase1 (Fig 2 F), QN-mRuby3 (Fig 4 H), Stb3 (Fig. S4 D), and Xbp1 (Fig. S4 B & C)) when transcription is exogenously activated, implying that their transcription, translation, and nuclear import machinery are fully functional.
2- Upregulation of carbohydrate storage enzymes: Fig. 1 K & L shows that budded Q-cells upregulate the main enzymes for carbohydrate energy storage as efficiently as unbudded Q-cells.
3- Upregulation of Autophagy: Fig. 4 F shows that High-Cdk1 Q-cells upregulated mature autophagosomes (as judged by mNeonGreen-Atg8 foci), which requires ATP during Atg8 activation (Reggiori and Klionsky, 2013).
4- No differences in the quantified mitochondrial signal: High-Cdk1 and Low-Cdk1 Q-cells have the same total mitochondrial fluorescence during starvation (Fig 4 J).

Instead of the depletion of mitotic factors or a general metabolic failure, our data shows that the accumulation of nuclear Xbp1 to high levels stops the cell cycle in High-Cdk1 cells during starvation. As explicitly explained now (See answer to reviewer 2 point 5, Video 6, and Fig. S4, G & H), *xbp1Δ* cells bypass the option of arresting in a High-Cdk1 state and continue with a risky cell cycle under stress. This shows that High-Cdk1 arrested cells could finish at least one extra division under stress, but Xbp1 prevents it. Whether Xbp1 causes the depletion of cell-cycle factors is a future research direction.

Reviewer 2 point 3: Sic1 levels are low in the high CDK1 quiescent cells, consistent with high CDK1 activity. It would be helpful to also characterize Far1 so that the behavior of all possible CKIs in established.

Far1 is not expressed in proliferating diploids (Chang and Herskowitz, 1990), which rules out its involvement in the cell cycle arrest of diploid strains. However, the reviewer's point is valid because nuclear Far1 has never been measured during quiescence entry. We, therefore, triggered quiescence in diploid and haploid strains carrying Cdc10-mCyOFP1 and Far1-mNeonGreen. As expected, diploid cells had a negligible Far1 nuclear signal in all Cdc10-clusters. In the haploid strain, Far1 was only accumulated in direct G1 Q-cells but decayed during the first six hours of starvation. Although Far1's stability control under starvation is outside the scope of the present work, these experiments show that Far1 is not accumulated in diploid or haploid High-Cdk1 Q-cells (Fig S1 F, shown here on the right).

Fig S1 F

Far1-mNeonGreen nuclear levels per Cdc10 cluster in haploid and diploid cells during quiescence entry
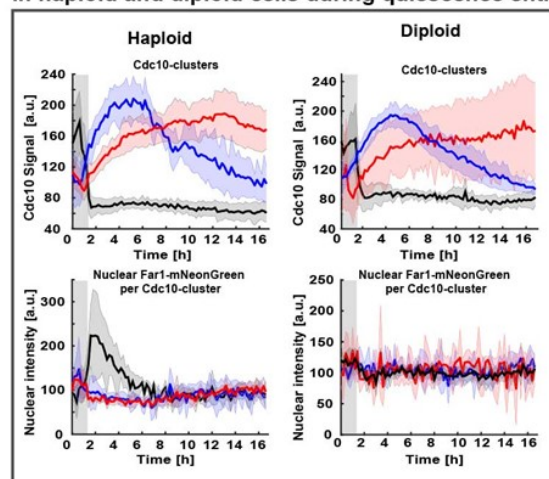
Reviewer 2 point 4: By only using a complete starvation arrest for the first few figures, it is particularly difficult to understand what the high CDK1 quiescent state is because it is not clear whether it would occur from a less extreme limitation. Providing information on additional arrest signals earlier in the manuscript could shed light on the nature of the state and how it relates to more traditional quiescence.

Exposure of proliferating cells to "*less extreme*" nutrient limitation is the focus of our 2018 Mol. Cell paper (Arguello-Miranda et al., 2018). We showed that exposure of proliferating cells to gradual starvation (1) does not produce an immediate proliferation arrest; cells divide for a couple of cell cycles before finally arresting (2) there are changes in daughter cells' size and in the length of the cell cycles under stress and (3) cells undergo remarkably different fates including quiescence, senescence, or meiosis and sporulation. Under such conditions, High-Cdk1 quiescent cells could be less than 1% of the population and only one of many different cell fates. The focus of the present study is on High-Cdk1 Quiescence entry in response to acute starvation.

The following papers offer descriptions of different gradual starvation conditions in which budded quiescent cells have been reported:

Johnston, G.C., J.R. Pringle, and L.H. Hartwell. 1977. Coordination of growth with cell division in the yeast Saccharomyces cerevisiae. Exp. Cell Res. 105:79–98. doi:10.1016/0014-4827(77)90154-9

Sudbery, P.E., A.R. Goodey, and B.L. Carter. 1980. Genes which control cell proliferation in the yeast Saccharomyces cerevisiae. Nature. 288:401– 404. doi:10.1038/288401a0
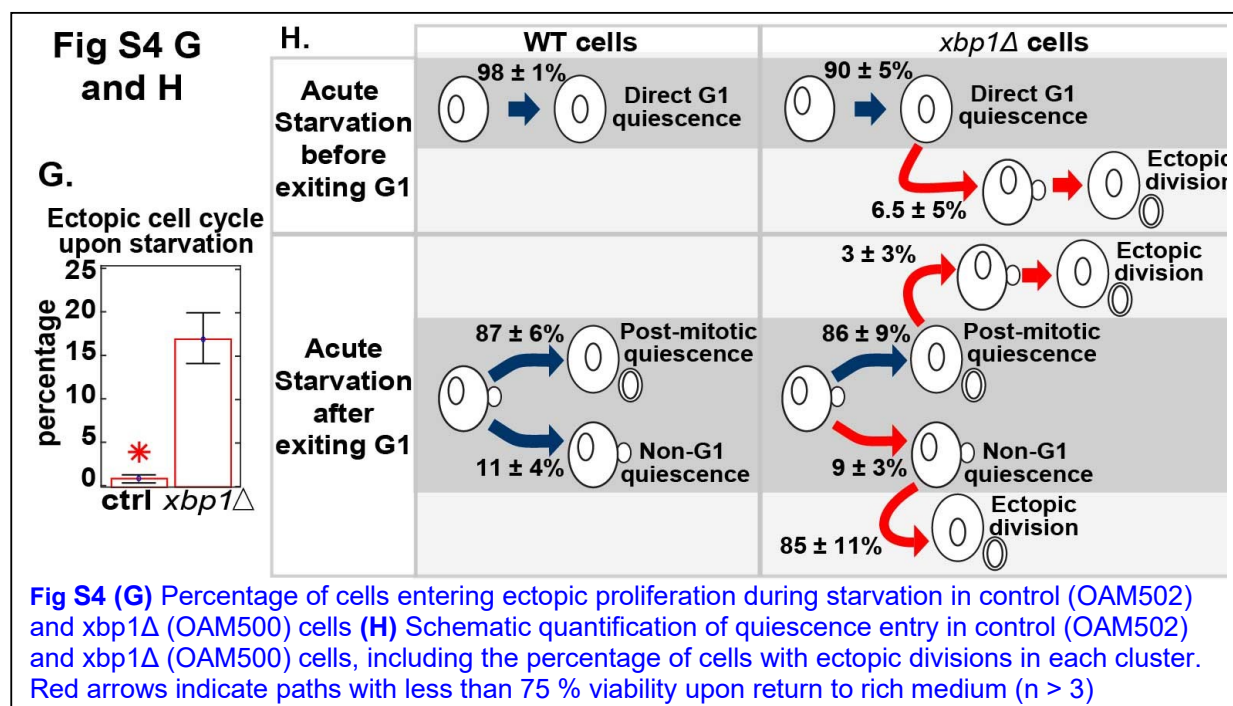
Allen, C., S. Büttner, A.D. Aragon, J.A. Thomas, O. Meirelles, J.E. Jaetao, D. Benn, S.W. Ruby, M. Veenhuis, F. Madeo, and M. Werner-Washburne. 2006. Isolation of quiescent and nonquiescent cells from yeast stationaryphase cultures. J. Cell Biol. 174:89–100. doi:10.1083/jcb.200604072

Sahin, A., B. Daignan-Fornier, and I. Sagot. 2008. Polarized growth in the absence of F-actin in Saccharomyces cerevisiae exiting quiescence. PLoS ONE. 3:e2556. doi:10.1371/journal.pone.0002556

Reviewer 2 point 5: XBP1 levels are associated with high G1 CDK quiescence and XBP1 inactivation results in fewer quiescent cells. The authors show that the viability of the quiescent cells is much lower when XBP1 is inactivated. Is it possible that expressing Xbp1 allowed the high Cdk1 quiescent cells to survive rather than inducing quiescence?

Thanks for requesting this clarification. In the previous version of the manuscript, we vaguely described the phenotype of *xbp1Δ* cells as: *"deletion of XBP1 led to altered patterns of cell cycle arrest during starvation"*. We have now extensively clarified the phenotype of *xbp1Δ* cells under starvation. We show that the primary defect of *xbp1Δ* cells is a failure to maintain cell cycle arrest in response to starvation. This affects a small portion of low-Cdk1 quiescent cells but has a devastating effect on High-Cdk1 Q-cells, which mainly die after engaging in cell cycle completion under stress. Fig. S4, G & H display the quantifications for all the paths taken by *xbp1Δ* cells and Video 6 shows now labeled examples of cells failing to maintain a high-Cdk1 arrest or undergoing ectopic cell division under starvation. The text has been modified to accommodate the added information:

"deletion of XBP1 led to altered patterns of cell cycle arrest during starvation with 16 ± 4 % of cells entering an ectopic, less viable cell cycle under stress (Fig. S4, G and H; Video 6). Comparison of cells that arrested for more than six hours under starvation in WT and xbp1Δ showed that 40 ± 10 % of WT cells remained arrested leading to High-Cdk1 quiescence, whereas most xbp1Δ cells bypassed entry into High-Cdk1 quiescence by completing a last division, with only 7 ± 5 % remaining arrested."

Fig S4 (G) Percentage of cells entering ectopic proliferation during starvation in control (OAM502) and xbp1Δ (OAM500) cells **(H)** Schematic quantification of quiescence entry in control (OAM502) and xbp1Δ (OAM500) cells, including the percentage of cells with ectopic divisions in each cluster. Red arrows indicate paths with less than 75 % viability upon return to rich medium (n > 3)

Xbp1 could be considered as a survival factor in the sense that it prevents a risky cell division under stress. To test other Xbp1's survival functions would require a quiescence-specific depletion system that allows cells to stably arrest in a quiescent state before triggering the destruction of Xbp1. Such system is not available in a microfluidics setup, yet.

Reviewer 2 point 6: Is it possible that another gene induces the high CDK1 quiescence cell cycle arrest, and without XBP1, the quiescent cells die, so XBP1 is a survival factor rather than a quiescence regulator?

The possibility that other factors induce High-Cdk1 arrest and that Xbp1 works exclusively as a survival factor predicts that *xbp1Δ cells* should remain arrested in a High-Cdk1 state with the same frequency as the WT, but with lower viability. This, however, is not the case: most *xbp1Δ cells* cannot remain arrested in a High-Cdk1 quiescence because they break free from the arrest and attempt to finish a risky cell cycle under stress. We now explicitly show this point in Fig. S4, G & H (see the previous answer).

Reviewer 2 point 7: Reviewer 3 point In Fig 8J, it looks like Stb3 levels are higher in the 2nd quiescence than the first, with a difference similar to that observed for Xbp1. This would seem to undermine the argument that Xbp1 alone is the crucial determinant of the cell's fate.

We thank the reviewer for pointing out that the nuclear levels of Stb3 significantly increase during a second quiescence entry. To clarify whether Stb3 plays a role in defining High-Cdk1 quiescence fate, we measured the increase of High-Cdk1 Q-cells after two consecutive quiescence entries in *stb3Δ*, *xbp1Δ,* and WT cells expressing Cdc10-mCyOFP1 as cell cycle marker. *STB3* deletion did not affect the increased accumulation of High-Cdk1 cells during the second quiescence entry, whereas *XBP1* deletion abolished the accumulation of High-Cdk1 cells (Fig 8 N, here on the right):

The results' text now includes these observations:

"Although Stb3 accumulated to higher levels during the second quiescence entry (Fig. 8 J), only Xbp1 persisted in rich medium after the first quiescence exit (Fig. 8 K) and accumulated to maximum levels 2 h ± 29 min faster during the second quiescence entry (Fig. 8 L). TPML Cdc10-clustering according to the first or second quiescence entry (Fig. S5 F) showed that high-Cdk1 Q-cells were indeed enriched during the second quiescence entry in WT (Fig. 8 M) and also stb3Δ cells, but not in xbp1Δ cells (Fig. 8 N)."


Fig 8 N.
Increase of high-Cdk1 Q-cells, 2nd quiescence entry

Other experiments showing that Stb3 is not associated with High-Cdk1 Q-cells are depicted below:
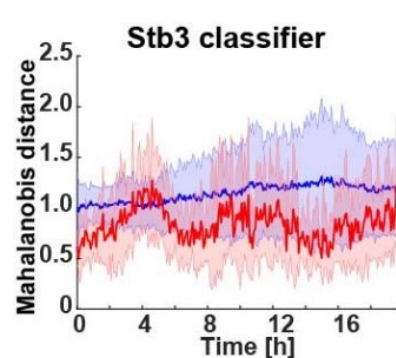
Fig S3 F: Stb3 nuclear levels have no predictive power as measured by a linear discriminant classifier (LDC)
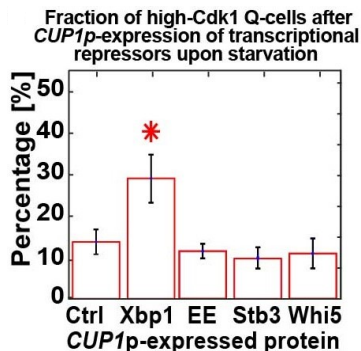
Fig 7 B: *CUP1*-expression of Stb3 during quiescence entry doesn't change the proportion of High-Cdk1 cells.
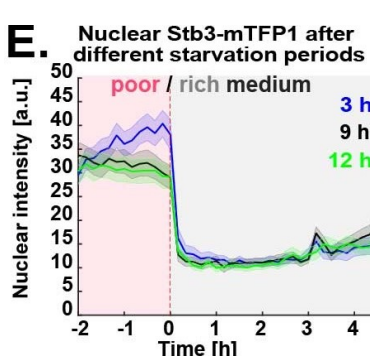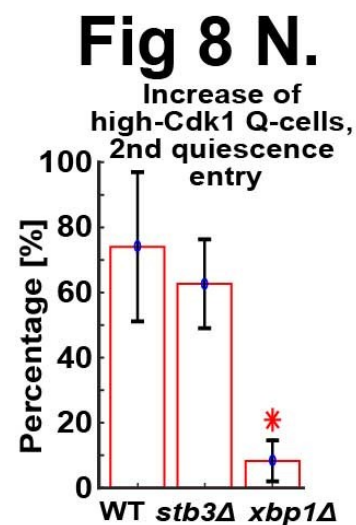
Fig S5 E: Accumulation of nuclear Stb3 does not show temporal integration of the starvation stimulus.

Reviewer #3: *In this paper by Arguello-Miranda et al, the authors show that acute starvation induce the emergence of 3 types of cells : direct G1, post-mitotic G1, and Non-G1 that respectively arrest in G1, finish their ongoing cell cycle and arrest in G1, or cease dividing as non-G1 cells. While the 2 first cell categories display a low Cdk1 activity, the latter has a high-Cdk1 activity as attested by several markers (persistent Cdc10 ring, no nuclear accumulation of Sic1 etc...), the cell types having the same survival rate. Then they show that high-Cdk1 Q-cells display an increased nuclear accumulation of Xbp1, Gln3, and Sfp1. They further show that high-Cdk1 quiescence was not defined by cell cycle stage at starvation onset but was rather determined by the stress status of the cell. They convincingly demonstrate that Xbp1 was a causative factor of high-Cdk1 quiescence entry as Xbp1 is sufficient to increase the frequency of high-Cdk1 Q-cells when accumulated at high levels (this effect requiring Xbp1 binding to DNA) and as the deletion of XBP1 reduced the total fraction and viability of high-Cdk1 quiescent cells. Finally, they show that Xbp1 may acted as an integrator of previous stress stimuli, which rendered the propensity of high-Cdk1 quiescence dependent on individual-cell history. This study is of great interest for the cell biologist community and overall, the data are very convincing.*

Reviewer 3 point 1- **1-Quiescent cells arrested in other cell cycle phases than G1 have been previously described in budding yeast by the Broek lab (Wei et al, 1993) and the Sagot lab (Laporte et al, JCB 2011 and eLife 2018). It was shown that these cells are arrested in the cell cycle as non-G1 cells (budded cells) and that individually, these cells are able to resume proliferation. The results presented here upon acute starvation confirm these previous findings in batch and clearly push them forward. Notwithstanding, the authors should clearly cite these previous studies in the introduction and further discussed them in the discussion section**

Thanks for pointing this out. Indeed, those are important studies that we have attempted to cite already:

In introduction: "In many organisms, however, cells can enter quiescence in states of high-Cdk1 activity (high-Cdk1 quiescence), which are essential for development and single-cell survival (Wei et al., 1993, Hajeri et al., 2005, Velappan et al., 2017, Baisch, 1988)."

In results: "To investigate the status of quiescence-associated processes (Sagot and Laporte, 2019) in low- and high-Cdk1 Q-cells, we induced starvation in Cdc10-mCyOFP1 strains containing fluorescent sensors for autophagy, protein aggregation, mitochondrial biomass, and DNA damage."

In addition, we now reference Broek's and Sagot's work more extensively in the following lines:

In introduction: High-Cdk1 quiescence occurs in mammalian oocyte development (Nixon et al., 2002), embryonic development of invertebrates (Hajeri et al., 2005, Nystul et al., 2003, Otsuki and Brand, 2018), plant meristems (Velappan et al., 2017), microorganisms (Laporte et al., 2011, Klosinska et al., 2011) and metastasis-initiating cancer cells (Wang et al., 2015).

In results: "To investigate the status of quiescence-associated processes (Sagot and Laporte, 2019, Laporte et al., 2018) in low- and high-Cdk1 Q-cells…"

In discussion: "Our results obtained under controlled microfluidic conditions confirm previous batch studies that described quiescence states in cell cycle stages other than G1 after depletion of nutrients in a carbon limited batch culture (Laporte et al., 2011) or by inducing cell cycle arrest before starvation (Wei et al., 1993)."

Reviewer 3 point 2- It would be interesting to compare the results previously published upon slow carbon exhaustion in YPD batch and pads and the results obtained here upon acute starvation of both carbon and nitrogen. To unable this comparison, the authors should clearly show the percentage of direct G1, post-mitotic G1, and non-G1 cells in the total population

Cdc10-cluster proportions are now explicitly reported in S Fig. 1 G (shown here on the right). Notice that previous works did not track the last cell division before quiescence entry in individual cells, which prevents any comparisons to the direct G1 and post-mitotic Q-cells described here. Regarding High-Cdk1 Q-cells, it is tempting to state that the ~7% of Non-G1 cells obtained under microfluidics conditions in this study corresponds to the ~ 10% fraction of budded quiescent cells measured in Fig 1 of Laporte et al., 2011 after gradual nutrient-depletion in a carbon-limited medium. However, both studies use different criteria: whereas the analysis of a time series for a Cdk1 activity marker defines Non-G1 Q-cells in this study, previously reported budded quiescent cells were classified according to a morphological feature (budding). We now acknowledge these parallels in the following sentence in results:
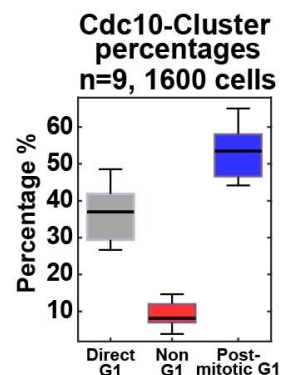

Fig S1 G

Cdc10-Cluster percentages
n=9, 1600 cells

"Strikingly, although most cells arrested in G1, as judged by maintaining an unbudded state, a reproducible minority of 7 ± 3 % cells remained arrested in a budded state (Fig. 1, G and H), which was highly viable upon return to rich medium (Fig. 1 I) and was reminiscent of budded quiescent states previously observed in stationary cultures in carbon limited medium (Laporte et al., 2011)."

Reviewer 3 point 3- Within the high-Cdk1/Non-G1 cells, they should also give the percentage of cells in S-phase (using the Cdc7 experiment of Fig2I) and the percentage of short spindle and anaphase B cells (using the Spc42 data of Fig2H).
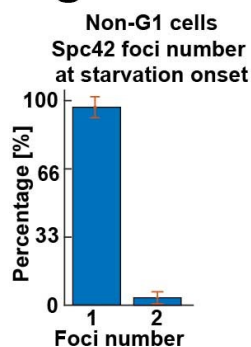
The nuclear signal of Cdc7 cannot be used to determine the percentage of cells in S-phase because nuclear Cdc7 is also enriched during G2 and metaphase thanks to its nuclear targeting subunit Dbf4, which is destroyed at anaphase and remains unstable during G1 (Cheng et al., 1999, Ferreira et al., 2000). We used the enrichment of nuclear Cdc7 "*to test whether Non-G1 Q-cells entered S-phase during starvation*". The nuclear levels Cdc7 in Non-G1 cells displayed in Fig 2I suggested that these cells could undergo DNA replication during starvation, which was confirmed in Fig 2 J by DNA staining.

Fig S1 J

Non-G1 cells
Spc42 foci number
at starvation onset

Fig S1 K

Non-G1 cells
Spc42 foci separation
after 20 starvation

As the reviewer suggests, Spc42 foci could better indicate cell cycle stage of Non-G1 Q-cells. We now present a detailed quantification of the SPB separation data in Fig. S1 J & K (shown here on the right). As expected from Fig 2 H, 96 ± 4% of High-Cdk1 Q-cells experienced starvation before SPB separation (Fig. S1 J) and exceedingly few High-Cdk1 Q-cells reached the SPB separation expected for metaphase or anaphase (>1.5uM, as judged by the spindle measurements defined by (Severin et al., 2001)). These data are consistent with the representative pictures in Fig 2 D, showing that High-Cdk1 Q-cells persist with a small bud.
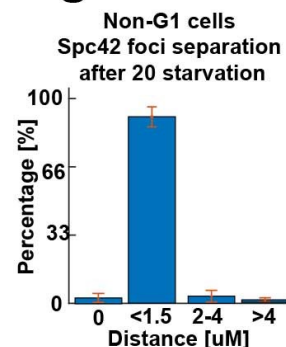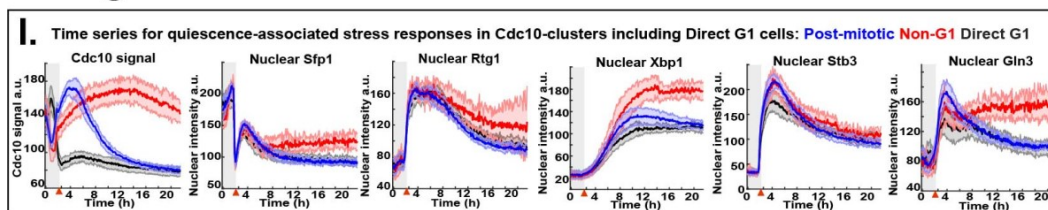
Reviewer 3 point 4- In the same vein, it should be interesting to know what king of cells undergo the one last division (the post-mitotic G1). It is all kind of cells or just the big mother cells that have already passed the critical size and the start point in G1?

The post-mitotic cluster is defined in the results section as cells that: "*were in a high-Cdk1 state at starvation onset, as judged by the presence of the septin ring*". The presence of a septin ring at the onset of starvation (Fig 2 D) shows that the post-mitotic cluster is exclusively composed of cells that passed the critical size threshold and crossed the START checkpoint before starvation onset. The destruction of Sic1 (Fig 1 E) and the inactivation of APC/C-Cdh1 (Fig 1 F & G) confirm this interpretation.
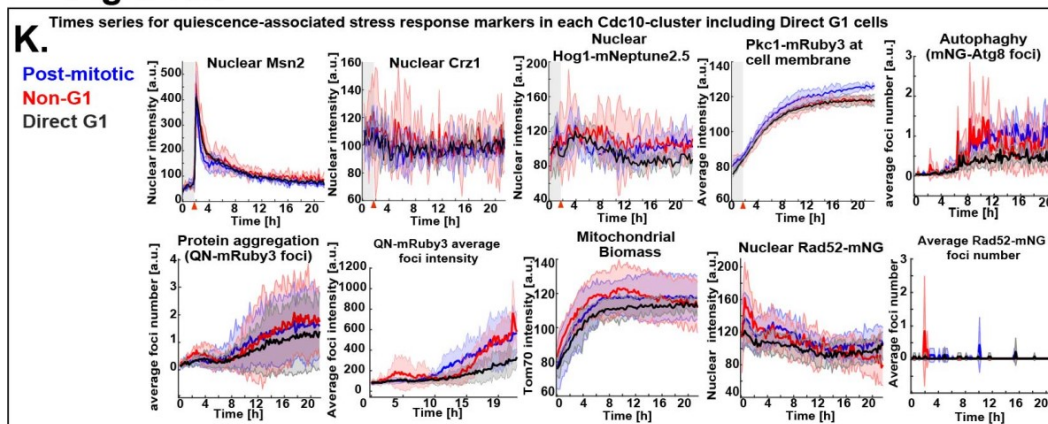
Reviewer 3 point 5- After Fig2, the authors focus on post-mitotic G1 (low-Cdk1 quiescence) and Non-G1 (high-Cdk1 qiescence). Why they do not show their results for direct G1? It would be very interesting to see what is the localization of the markers of Fig3 and Fig4 in these cells. Are direct G1 displaying autophagosomes? Normal mitochondria? Q/N aggregates? Rad52 foci? Etc... Of note, Laporte et al (JCB 2011), have shown that actin bodies, proteasome granules and mitochondria reorganized identically in both budded and unbudded quiescent cells. This should be mentioned when describing Fig4.

As the reviewer points out, the main focus of our work is the comparison of post-mitotic and Non-G1 Q-cells. However, we agree with the reviewer that the data for direct G1 cells should be included in the paper as a reference for future single-cell studies on quiescence. We now display the results for direct G1 cells in Fig. S2 I & K, which include all Cdc10-clusters and the explicit mention: "*including direct G1 Q-cells*" (see below). We did not include this information in the first version of the paper because stress responses and quiescence-associated processes have no significant difference between direct G1 cells and post-mitotic cells, consistent with Laporte et al. 2011.

Fig S 2I.

I. Time series for quiescence-associated stress responses in Cdc10-clusters including Direct G1 cells: Post-mitotic Non-G1 Direct G1

Fig S 2K.

K. Times series for quiescence-associated stress response markers in each Cdc10-cluster including Direct G1 cells

Reviewer 3 point 6- Furthermore, the images shown in Fig3 and Fig4 are of very poor quality. Probably because the authors utilized a 40X 1.3 NA objective. Can the author use a better objective? Would a 63X 1.4NA be compatible with the Cell Asics setting? If not, the authors should present images to convince the reader about of the observed changes in localization. For examples, in Fig 3, I do not see any Sfp1 cytoplasmic clusters appearing, etc...; in Fig4, mitochondria look like clumps (actually, the authors should explain how did they measure what they call mitochondria "biomass", I do not see any Rad52 foci but rather a blurry fluorescent nucleus.... Showing better quality images and statistics for the 3 cell categories (direct G1, post-mitotic G1, and non-G1) would greatly improve the data.

The images used in Figs 3 and 4 are not aesthetically pleasing because they are optimized for algorithmic or machine learning quantification of single-cell features as explained in the material and methods section *"Image processing and quantification of cellular features"* and in the freely available code deposited in GitHub. For instance, when a human brain looks for foci, it looks for dot-like pixel groups brighter than the background in a subjective manner. In contrast, the algorithms used in this study look for a cluster of pixels that can be mathematically described as a gaussian function and are independent of filtering with a 3 x 3 structuring element. This approach depends on pixel patterns that can be obtained from a 40X 1.3 NA objective and maximizes the number of cells that can be simultaneously imaged while retaining the features for statistical analysis, as has been shown in previous works (Doncic et al., 2011, Doncic et al., 2013, Arguello-Miranda et al., 2018, Wood and Doncic, 2019, Schmoller et al., 2015).

Mitochondrial biomass is defined in the results section: *"Total mitochondrial biomass, as measured by the mean fluorescent intensity of the translocase Tom70 (Hughes et al., 2016)…"* . Examples of algorithmically detected Rad52 foci are labeled with arrows in Fig. S2 N.

Reviewer 3 point 7- In Xbp1 delta cells, it is of critical interest to give the % of each cell category (direct G1, post-mitotic G1, and non-G1) and their respective viability (Fig7I show that there is very little high-Cdk1 cells in a Xbp1 delta population and that these cells massively die upon starvation. What is the viability of the Xbp1 delta low-cdkA cells?

We now show the % viability of low-cdk1 Q-cells for the Xbp1 delta strain in Fig S4 I (shown here on the right), which indicates that *XBP1* deletion does not significantly affect the viability of quiescent cells that reached a stable low-Cdk1 arrest.

Fig S4 I. Viability of clusters that reached Low-Cdk1 quiescence
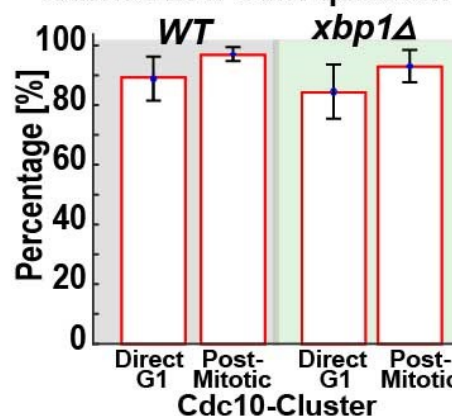
Reviewer 3 point 8- They author claim that Xbp1 acted as an integrator of previous stress stimuli, which rendered the propensity of high-Cdk1 quiescence dependent on single-cell history. Although very appealing, the text should be soften given the fact that Xbp1 "history" is lost after 4h in rich medium (Fig. 8H). In fact, the cumulative effect observed in Fig8 E and

F is due to the short period of nutrient replenishment between two starvations. Cells have hardly the time to re-enter the division cycle and divide once....so given Fig 8B, data obtained in 8E and F are trivial. Fig8K is more convincing, but again 8h hours of nutrient replenishment may hardly allows 4 divisions, not enough to dilute Xbp1. In the text the conclusions have to be soften accordingly.

The concept of single-cell history depicted in Fig 8 C follows the single-cell memory definition proposed by Bruce Morimoto and Daniel Koshland in Fig 1 A & B of their 1991 FASEB paper (Morimoto and Koshland, 1991). Below we contrast the two figures. Koshland's (black and white) Fig 1 A is equivalent to the "case 2" in Fig 8 C (in color), whereas Khosland'Fig. S1 B is equivalent to the "case 1" in Fig 8 C:
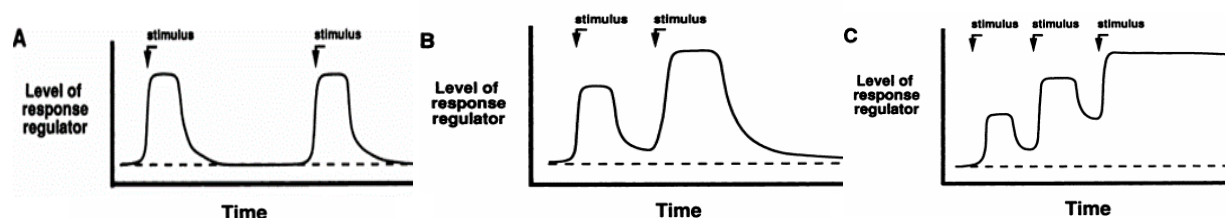
Figure 1. Schematic illustration of short- and long-term memory in organisms or individual neurons. *A)* A stimulus generates a transient response in which the response regulator changes level for a finite period and returns to the initial value. As the state of the response regulator at the time of a second stimulus is the same as before the first stimulus, the second stimulus gives an identical response. Thus only short-term memory is observed. *B)* The second stimulus in this example is received before the first response has returned to zero. Hence the response regulator does not follow exactly the same course as previously. As shown here, it may take longer to return to zero, but if it does so, the memory is erased and an identical combination of stimuli will give an identical response. *C)* Three successive stimuli raise the response regulator over a threshold value so that it remains at a high level essentially indefinitely. Any new stimuli will therefore impinge on a different situation with the response regulator at a high initial level. Long-term memory has been established.

Fig 8 C.
Assay to assess stress signal integration by nuclear accumulation of stress factors
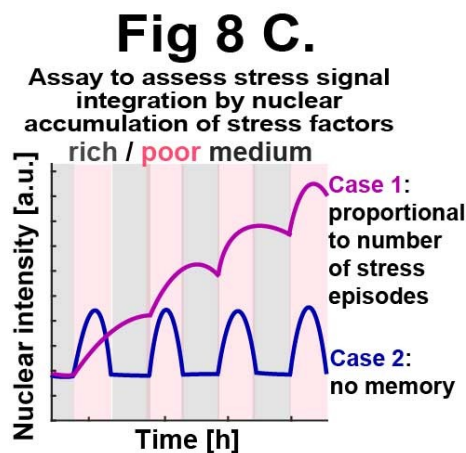
Koshland's concept of memory through temporal signal integration was expanded by the work of Erin O'Shea's group, which described a quantitatively rigorous definition of a biochemical signal integrator in Fig 2 of their Hao, et al. 2013, Science paper (Hao et al., 2013). These works show that the definition of memory in single cells does not imply a precise time scale but is instead associated with the kinetics by which an input signal is turned into an output signal. In other words, there is not a precise number of cell cycles required to label a process as having "memory". Accordingly, the Spencer and Meyer laboratories have shown cellular memory for processes involving a single cell division, for instance, by explaining how mitogen signals or replication stress in mother cells control daughter cells' fate in the next cell cycle (Arora et al., 2017, Min et al., 2020, Yang et al., 2017).
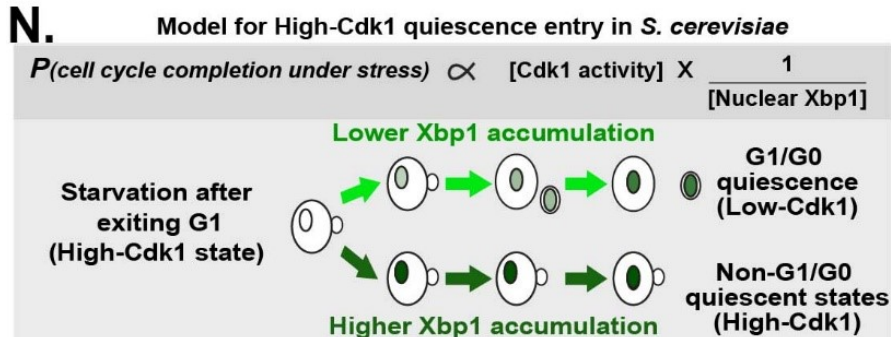
Thus, when the reviewer states that *"8h hours of nutrient replenishment may hardly allow 4 divisions, not enough to dilute Xbp1",* it is actually remarkable that information from a previous quiescence entry persists for at least 3-4 cell divisions in the nuclear levels of Xbp1.
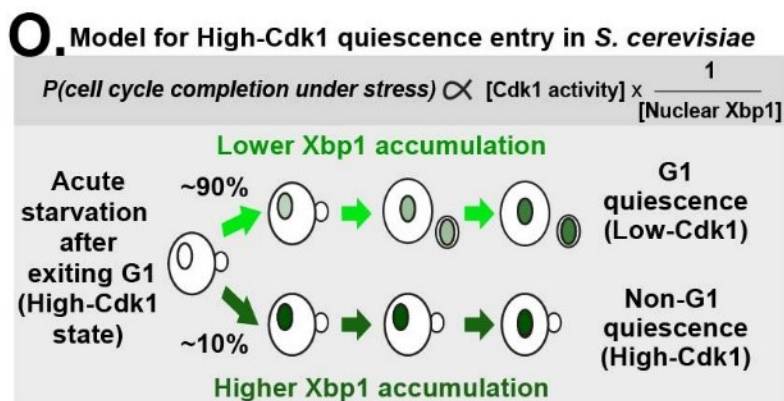
The next 2 points address figure 8 N:
Reviewer 3 point 9- Importantly, given that high-Cdk1 cells hardly represent 10% of the total cell population, the authors should add the proportion of each cell types in a WT population in the model presented in Fig8N (90% next to low cdk, 10% next to high cdk).
Reviewer 3 point 10- The use of the term G0 in the text and Fig8N is not appropriate, especially if cells are budding.

Fig 8N now includes the percentage of cells in each fate and the G0 term has been removed. The former Fig. 8N:

N. Model for High-Cdk1 quiescence entry in *S. cerevisiae*

$$P\text{(cell cycle completion under stress)} \propto [\text{Cdk1 activity}] \times \frac{1}{[\text{Nuclear Xbp1}]}$$

Becomes:

O. Model for High-Cdk1 quiescence entry in *S. cerevisiae*

$$P\text{(cell cycle completion under stress)} \propto [\text{Cdk1 activity}] \times \frac{1}{[\text{Nuclear Xbp1}]}$$

Reviewer 3 point 11- In the last sentence of the introduction: "Our results show that cell cycle independent integration of stress stimuli by transcriptional repressors is a viable cellular strategy to establish quiescence outside of a G1/G0 state", the authors utilize the term "strategy". In this study, it just happens that Xbp1 accumulates upon repetitive acute starvation - so OK for the term "integrator" of past stress signals. Yet, the "integration" is rapidly lost by dilution after a few divisions... The consequent arrest as non-G1 cells cannot be defined as a STRATEGY. At this step, it is just an observation, not a specific cell "program". Thus, this specific part of the text has to be soften to better fit with the conclusions from the experiments.

The usage of the word strategy has been corrected in the text. The sentence:
*"Our results show that cell cycle independent integration of stress stimuli by transcriptional repressors is a viable cellular **strategy** to establish quiescence outside of a G1/G0 state."*

Becomes:
*"Our results show that cell cycle independent integration of stress stimuli by transcriptional repressors is a viable cellular **response** to establish quiescence outside of a G1/G0 state."*

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September 23, 2021

RE: JCB Manuscript #202103171R

Dr. Orlando Arguello-Miranda
The University of Texas Southwestern Medical Center
Lyda Hill department of Bioinformatics
6000 Harry Hines Blvd.
NL6.120B
Dallas, Texas 75390

Dear Dr. Arguello-Miranda:

Thank you for submitting your revised manuscript entitled "Cell cycle-independent integration of stress signals promotes Non-G1/G0 quiescence entry". We are happy to say that the reviewers are now supportive of publication. Please be sure to address any remaining concerns in the final version of the manuscript. Pending these revisions and any revisions necessary to meet our length and other formatting guidelines (see details below), we would be happy to publish the paper in JCB.

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

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Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

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Sincerely,

Tobias Meyer
Monitoring Editor
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Scientific Editor
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Reviewer #1 (Comments to the Authors (Required)):

The authors properly answered my questions, went into details by addressing comments by me and the other two referees.
The main text did not change much as a result of these, but the supplement shows more thoughtful results now.

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

The authors have addressed my comments and the comments from the other reviewers thoroughly. I have no outstanding concerns.

Reviewer #3 (Comments to the Authors (Required)):

The authors have significantly improved the manuscript and convincingly replied to the comments raised. Experiments in stb3delta cells are particularly conclusive. Providing data on xbp1delta cell proportion within the population (SupS4I) and cell viability also very much strengthens the authors conclusions. I also very much appreciated the addition of data concerning the G1 cells in new Sup S1G and Sup S2I & K and the scoring of Spc42 localization (Sup S1J & K). The model in figure 8 has also been satisfactorily improved.
I've just seen one error in FigS1K the h for hour is missing (Should be 20h instead of 20).

For all those reasons, I think this version of the manuscript is now suitable for publication.