CDK actively contributes to establishment of the stationary phase state in fission yeast

Motoaki Hiraoka, Yuki Kiyota, Shinnosuke Kawai, Yusuke Notsu, Kohei Yamada, Katsuyuki Kurashima, Jing-Wen Chang, Shunsuke Shimazaki and Ayumu Yamamoto
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AUTHORS: Motoaki Hiraoka, Yuki Kiyota, Shinnosuke Kawai, Yusuke Notsu, Kohei Yamada, Katsuyuki Kurashima, Jing-Wen Chang, Shunsuke Shimazaki, and Ayumu Yamamoto
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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In the study by Hiraoka et al, the authors characterize fission yeast cells in stationary phase and those subjected to acute glucose depletion (GD). They find that, when the cells enter stationary phase, the sizes of the nuclei and chromosome-occupying space decrease, frequency of sister chromatid separation is reduced, and Brownian-like fluctuation of chromosomes are repressed. These changes are less evident in GD cells, and require fully-functional CDK before/upon the entry into stationary phase. When the cells enter stationary phase, intracellular localization of CDK changes dependent on its kinase activity. Concomitantly, chronological life span of the stationary phase cells is significantly longer than GD cells, and this extension of life span requires fully-functional CDK. From these observations, the authors conclude that the CDK activity in log phase is required to change physiological state in subsequent stationary phase, and that such changes are important for survival of stationary phase cells.

While their findings are potentially important and interesting, their conclusions are not undoubtfully supported by only the results shown in the current manuscript. Additional experiments and further clarification of the logic leading to the conclusions are required.

Comments for the author

Major points:
While non-proliferative cell cycle stages induced by glucose- or nitrogen-depletion and stationary phase are collectively referred as ?quiescence,? this terminology is quite confusing in this manuscript. Non-proliferative state by glucose-depletion, that by nitrogen-starvation and stationary phase are distinct states, and it appears illogical to consider them as same or closely-related biological phenomena. Indeed, the results shown in this study clearly indicate that physiological status in GD cells and stationary cells are different. In this view, abstract and introduction seem misleading, as it gives the impression that cellular ?quiescence? would be characterized collectively in this manuscript, but, in fact, only the stationary phase and GD are characterized.

page 2 line 8, page 3 line 15: While the authors state that quiescent cells are metabolically inactive, this statement appears inconsistent with the results shown in Figs. 4A and B. Stationary phase cells appear to use nutrients other than glucose to extend their chronological life span.

page 7 lines 11-12, Figs. 4 and 5: To show the reduction of sizes of the nucleus / chromatin region, which may be consistent with a previous report demonstrating shrinkage of chromatin structure in dormant G0 cells (doi.org/10.1242/jcs.109.6.1347 and Sajiki et al 2009), the authors measured their area in two-dimensional images, but this method appears inaccurate. As the nucleus is a spheroid, the area of its cross-section greatly changes depending on the position of the focal plane. Authors should measure their volume using three-dimensional reconstructed images.

page 8 lines 9-10: While the authors suppose that glucose exhaustion induces entry into stationary phase of cells grown in glucose-rich medium, it is not self-evident. Various aspects of stationary phase, such as high cell density accumulation of toxic compounds in medium and depletion of nutrients other than glucose, can potentially inhibit cellular proliferation. The authors may confirm this supposition by examining whether cells in stationary phase can resume cell division upon replenishment of glucose.

Figs 2B and C: Sister chromatid separation at a high frequency during log and stationary phases are unexpected, and thus very interesting. It may be better to confirm this result by other methods, such as fluorescent in situ hybridization to deny a possibility that observed chromatid separation is not due to experimental artifacts caused by introduction of the lacI-GFP system.

Figs. 2E and 5G: While the authors estimate fluctuation mobility of the loci by plotting the mean square displacement (MSD), changes in the MSD does not necessarily reflect changes in mobility of the locus. MSD simply represents the amount of displacement for given time. Because motion of the chromosomal locus is restricted within the inside of the nuclear envelope, the sizes of the nuclei supposedly determine the upper limit of the displacement. Thus, downshifts of MSD plots in stationary phase cells may just reflects reduction of the size of the nucleus, but not fluctuation mobility. Likewise, an upward shift of MSD in cdc2-L7 (page 11 line 15) may be caused by the bigger nucleus in the mutant than that in the wild-type.

Figs. 5D, E and F: Since the cell length, the sizes of nuclear area and those of chromosome area are larger in cdc2-L7 mutant cells growing in log phase at 32 C than those in the wild-type cells (page 15 lines 14-15), significant increase of them in the mutant cells in stationary phase does not necessarily indicate that the CDK activity is required for changes in these size and length upon the entry into stationary phase. Although these sizes are significantly larger in the mutants than in the wild-type cells in stationary phase, they are obviously smaller in the mutant cells in stationary phase than those in log phase indicating reduction of these sizes occur upon the entry into stationary phase even in the absence of wild-type CDK. The authors should calculate the ratio of these sizes between log phase and stationary phase cells, and compare these ratio between the wild-type and the mutant cells.

Reviewer 2

Advance summary and potential significance to field

The primary finding of this study is that CDK plays a significant role in establishing a stationary cell state. The authors described the detailed phenotypes of stationary cells, which is mainly consistent with previous reports. They showed that these phenotypes largely depended on CDK activity at the entry of the stationary phase using CDK mutant. Although this work suggests the CDK role in stationary entry only in the fission yeast cell, a simple model that has only one CDK, these findings should be the first step to investigating evolutionarily conserved CDK contribution for the stationary phase and also for the other dormant states in different organisms.

Comments for the author

Overall, the current manuscript is well-written, and the results are clearly presented. However, there are still some concerns listed below that the authors need to clarify. I do not ask for any additional experiments and the author might need to do some data analysis and rewrite the manuscript.

1. Statistics analyses are mostly well described. However, the presentation is yet inadequate on some points. In Fig8A-D, there is no explanation for statistics. If the description of statistics for Fig8E is also applied to these data, the authors should use some multiple comparison method because they would repeatedly test in multiple time points. If the authors would like to focus on the specific time point, they should explain the rationale. Instead, the authors might use the other statistical test, which can directly compare the viability curve, such as log-rank test, rather than using multiple t-test.

2. The amount of CDK in the nucleolar should be quantified in Fig7 because the localization of CDK in the nucleus and nucleoli does not seem mutually exclusive, but rather there is some residual nucleoli localization with analog and residual nuclear localization in control as well. Some quantitative characteristics, such as nucleoli signal intensity normalized by that of the the nucleus should be useful to compare the localization changes in addition to the categorical classification in Fig7B.

3. I wondered why cdc2-L7 phenotypes in the stationary phase were already apparent in the log phase cell (Fig5D-G). The authors need to supply rational excuses for this phenomenon. I guess that cell length increment and nuclear expansion in log phase cdc2-ts cell might be explained by just the delay in the cell cycle progression. But, I am not sure why the chromosome area, mobility and separation were affected by CDK inactivation in the log phase.

4. The authors performed conditional CDK inactivation before and after the establishment of the stationary phase in Fig8C to distinguish whether CDK is required for either the establishment or maintenance of the stationary phase.
The result apparently suggested that CDK requires sole for establishment.
However, I have a concern regarding the discrepancy between Fig8B and Fig8C in respect of the phenotype severity. If CDK is required only for the establishment, the phenotype in Fig8C and Fig8B should be comparable, but conditional knockdown before the establishment of the stationary phase

does not affect so much. The authors should explain in the manuscript why the effect of CDK inhibition before the stationary phase is subtle.

Minor suggestions:
1. On page 15, line 13, "The separation frequencies of ~". The authors clarify the comparison in this sentence, like "The separation frequency of cdc2-L7 cells increased compared to WT".

2. Too low resolution Figure S5.

First revision

Author response to reviewers' comments

Reviewer 1 Comments for the Author:
Major points:
Comment 1:
While non-proliferative cell cycle stages induced by glucose- or nitrogen-depletion and stationary phase are collectively referred as "quiescence", this terminology is quite confusing in this manuscript. Non-proliferative state by glucose-depletion, that by nitrogen-starvation and stationary phase are distinct states, and it appears illogical to consider them as same or closely-related biological phenomena. Indeed, the results shown in this study clearly indicate that physiological status in GD cells and stationary cells are different. In this view, abstract and introduction seem misleading, as it gives the impression that cellular "quiescence" would be characterized collectively in this manuscript, but, in fact, only the stationary phase and GD are characterized.

Our response:
As we stated in the beginning of the Introduction section, quiescence is the non-dividing, but proliferation-competent state. In this definition, "quiescence" comprehensively includes stationary phase, and we believe that there is no problem in terminology. However, it is also true that quiescence states induced by distinct nutrient depletion or in different organisms are not completely the same, as pointed out by the reviewer. In the previous version, our statement may make readers think that all the quiescent states are the same since we often described "stationary phase" as "quiescence". Considering these and the reviewer's criticism, in the revised version, we avoided using "quiescence" for describing "stationary phase" and focused more on stationary phase in yeast in the Abstract and the Introduction sections by removing most of description about quiescence in other organisms. According to the changes in the Introduction, we discussed similarities among different quiescent states in the Discussion section. Major changes are following.

1) Abstract
We used only stationary phase and eliminated the term, "quiescence". Specifically, to focus on stationary phase, we also changed the first sentence as follows.

Line 7 on Page 2
"Upon exhaustion of essential environmental nutrients, unicellular organisms cease cell division and enter stationary phase,..."

In addition, we edited Abstract according to changes in the manuscript described below.

2) Introduction
We reorganized the second, third, and fourth paragraphs (from line 1 on page 4 to line 18 on page 5) and focused solely on stationary phase in yeast by removing description about quiescence in mammalian cells. The removed sentences in the old version are following.

Line 22 on page 3 in the old version
"To enter quiescence, cells must exit the cell cycle. Consistently, quiescence is characterized by the accumulation of factor(s) that inhibit the master cell cycle regulator, cyclin-dependent kinase

(CDK) (Daga et al., 2003; Marescal and Cheeseman, 2020; Matson and Cook, 2017; Pajalunga et al., 2007; Sun and Buttitta, 2017; Tesio and Trumpp, 2011; Valcourt et al., 2012; van Velthoven and Rando, 2019; Velappan et al., 2017). However, quiescence is not merely a consequence of cell cycle arrest. Arrest of the cell cycle by forced expression of CDK inhibitors does not induce quiescence phenotypes in mammalian cells (Coller et al., 2006)."

Line 21 on page 4 in the old version
"Many studies have shown that quiescence-associated changes take place not at the time of quiescence entry but in the preceding cell cycle. Mammalian cells that enter the quiescent state exhibit lower CDK activity in the preceding cell cycle than those that continue to cycle (Adikes et al., 2020; Arora et al., 2017; Barr et al., 2017; Fan and Meyer, 2021; Spencer et al., 2013)."

3) Discussion
We discussed similarities among distinct quiescent states in the "Conclusion" section as follows.

Line 21 on page 24
"It is currently unclear whether CDK actively contributes to quiescence induced by distinct nutrient depletion or in other organisms. This is mainly because CDK inhibition is essential for the establishment of quiescence in all cases (Barbet et al., 1996; Daga et al., 2003; Marescal and Cheeseman, 2020; Matson and Cook, 2017; Moreno-Torres et al., 2015; Pajalunga et al., 2007; Sun and Buttitta, 2017; Tesio and Trumpp, 2011; Valcourt et al., 2012; van Velthoven and Rando, 2019; Velappan et al., 2017; Werner-Washburne et al., 1993; Yanagida, 2009; Zinzalla et al., 2007)."

Comment 2:
page 2 line 8, page 3 line 15: While the authors state that quiescent cells are metabolically inactive, this statement appears inconsistent with the results shown in Figs. 4A and B. Stationary phase cells appear to use nutrients other than glucose to extend their chronological life span.

Our response:
We changed the description as follows.

Line 8 on page 2
"a metabolically repressed state"

Line 15 on page 3
"Quiescent cells are characterized by the substantial repression of their metabolic activity and their resistance to various stresses,..

Comment 3:
page 7 lines 11-12, Figs. 4 and 5: To show the reduction of sizes of the nucleus / chromatin region, which may be consistent with a previous report demonstrating shrinkage of chromatin structure in dormant G0 cells (doi.org/10.1242/jcs.109.6.1347 and Sajiki et al 2009), the authors measured their area in two-dimensional images, but this method appears inaccurate. As the nucleus is a spheroid, the area of its cross-section greatly changes depending on the position of the focal plane. Authors should measure their volume using three-dimensional reconstructed images.

Our response:
In the revised version, we have measured volumes of the cell and the nucleus in wild-type and *cdc2* mutant cells. To measure the nuclear volume, we used images of the nuclear membrane. However, the nuclear membrane was clear only in the right section of the nucleus, and in most of the serial sections, the nuclear membrane was not clear due to its oblique section. Since it was difficult to detect the nuclear outline in the serial sections, we did not obtain the nuclear volume using three-dimensional reconstructed nuclear images. Instead, we measured the nuclear volume by regarding the nucleus as a spheroid with the semi-axis lengths, a, b, and $(a + b)/2$, where a and b are the lengths of the major and the minor axes of an ellipse fitted to the nuclear membrane seen in the right section of the nucleus. The nuclear shape in *S. pombe* is close to sphere, and thus our measurement is fair enough to get the right size of the nucleus. Indeed, our results are consistent with the previous reports by other groups (Lemiere et al, 2022; Kume et al, 2019). The

measurement of the nuclear volume confirmed a significant reduction in volume of the nucleus in stationary phase and GD cells. These new results are shown in Figs. 1D, 5E, 5J and Fig S5E.

It is currently impractical for us to precisely measure the volume of the chromosome- occupying space using three-dimensional reconstructed chromosomal images due to difficulties in precise determination of the chromosomal area in serial sections. In the projection image, we generated binarized chromosomal images in such a way that the binarized chromosomal region fits with nuclear region. However, it was difficult for us to generate precise binarized chromosomal regions in the serial sections where the nuclear regions were unclear. Due to this difficulty, we could not measure the chromosomal volume in a precise and reliable manner. Therefore, we did not show chromosomal volumes in the revised manuscript. However, we believe that area measurement is enough to draw our conclusion that chromosomal volume decreases. The chromosomal to nuclear area ratio was not shown since it is not informative compared to the volume ratio. Finally, we cited Su et al (1996) as a reference for nuclear/chromosome shrinkage as suggested by the reviewer (line 8 on page 6, and line 8 on page7).

Comment 4:
page 8 lines 9-10: While the authors suppose that glucose exhaustion induces entry into stationary phase of cells grown in glucose-rich medium, it is not self-evident. Various aspects of stationary phase, such as high cell density, accumulation of toxic compounds in medium and depletion of nutrients other than glucose, can potentially inhibit cellular proliferation. The authors may confirm this supposition by examining whether cells in stationary phase can resume cell division upon replenishment of glucose.

Our response:
We agree that nutrients other than glucose is reduced or depleted in stationary phase. Indeed, we have already found that replenishment of glucose alone is not sufficient for resumption of the cell cycle of stationary phase cells and that addition of other nutrients is required for cell cycle resumption (manuscript in preparation). In addition, it was reported that *S. pombe* cells secrete fatty acids, which affect cell growth (Sun et al, 2016). Therefore, we cannot exclude the possibility that depletion of other nutrients and/or the presence of unidentified secreted compounds contribute to the establishment of the stationary phase state. According to the reviewer's criticism, we describe this possibility in the Discussion section as follows.

Line 6 on page 20
"However, depletion of nutrients other than glucose induces quiescence (Klosinska et al., 2011) and *S. pombe* cells secrete compounds that affect cell growth (Sun et al., 2016). Thus, although glucose depletion is a major trigger of stationary phase, we cannot completely exclude the possibility that depletion or decline of other nutrient(s) and/or the presence of unidentified secreted compounds additionally contribute to stationary phase establishment and that the GD state is different from the stationary phase state due to lack of these additional effects."

Comment 5:
Figs 2B and C: Sister chromatid separation at a high frequency during log and stationary phases are unexpected, and thus very interesting. It may be better to confirm this result by other methods, such as fluorescent in situ hybridization, to deny a possibility that observed chromatid separation is not due to experimental artifacts caused by introduction of the lacI-GFP system.

Our response:
Fluorescence in situ hybridization (FISH) is not easy analysis and often gives varieties in results. Furthermore, considering the small separation frequencies, and varieties in the frequencies among observations, we think it is very difficult to prove sister chromatid separation and its stationary phase- associated repression by this method. Considering these, we did not carry out FISH analysis. Instead, we stated the possibility raised by the reviewer and softened our statement in the Results section as follows.

Line 13 on page 9
“Although we cannot completely exclude the possibility that sister locus separation and its repression are artifacts generated by the *lacI*/*lacO* chromosome visualization, these results suggest that the chromosomal association state is distinct in stationary phase.”

Line 17 on page 9
Changed “indicating” to “suggesting”.

Comment 7:
Figs. 2E and 5G: While the authors estimate fluctuation mobility of the loci by plotting the mean square displacement (MSD), changes in the MSD does not necessarily reflect changes in mobility of the locus. MSD simply represents the amount of displacement for given time. Because motion of the chromosomal locus is restricted within the inside of the nuclear envelope, the sizes of the nuclei supposedly determine the upper limit of the displacement. Thus, downshifts of MSD plots in stationary phase cells may just reflects reduction of the size of the nucleus, but not fluctuation mobility. Likewise, an upward shift of MSD in cdc2-L7 (page 11 line 15) may be caused by the bigger nucleus in the mutant than that in the wild-type.

Our response:
Given spatial scales of the fluctuation, it is unreasonable to interpret that the observed differences in chromosome fluctuation are caused by the different nuclear sizes. To confirm this, we regarded the chromosome locus as a particle and conducted simulations of the particle diffusing in confined spherical spaces that reflect the observed nuclear sizes in log and stationary phases. In our simulations, the MSD plots of the particle diffusing in the confined distinct spherical spaces exhibited the same kinetics until the MSD plots exceed 0.1 μm^2, which is consistent with the order of the nuclear radii. Therefore, the repression of chromosome fluctuation cannot be simply attributed to the spatial confinement. The result of the simulations is now shown in Fig. S2B and stated as follows.

Line 20 on page 10
“Considering the spatial scales of the fluctuation, this fluctuation repression cannot be attributed simply to spatial confinement. This conclusion is supported by simple numerical simulations of a particle diffusing in confined spherical spaces, which reflect the observed nuclear sizes in log and stationary phases (Fig. S2B).”

In the legend of Fig. S2B
“(B) MSD plots obtained from numerical simulations of a particle diffusing in confined spherical spaces. The spaces reflect the observed nuclear sizes in log (R_{nuc}= 1.238 μm) and stationary (R_{nuc}=0.925 μm) phases. The effect of the nuclear size on MSD is appreciable only when MSD exceeds 0.1 μm^2, which is not in the observation range in Fig. 2D and reasonable for nuclear radii that are in the order of 1 μm. Note that this simulation indicates that the repression of chromosome fluctuation cannot be simply attributed to the nucleus size-dependent spatial confinement. However, this does not exclude the possibility that nuclear size affects the chromosome fluctuation indirectly, i.e., nuclear shrinkage causes some changes in the intranuclear molecular composition through an uncharacterized biochemical mechanism, which lead to the repression of chromosome fluctuation.”

The simulation method is described in the Materials and Methods section as follows.

Line 9 on page 34

“Numerical simulation of chromosome fluctuation with spatial confinement
A chromosomal locus was regarded as a particle diffusing in a space surrounded by a spherical wall representing the nuclear envelop. The time step of the simulation was taken to be Δt = 0.5 *s*. Correlated random numbers were generated to make the displacement of the particle at each step. The statistics of the random displacement is such that MSD(t) αt^{α}, where the exponent is set to be *a* =0.67 in order to reproduce the experimental results. Generation of correlated random numbers was carried out using the Fourier transformation method (Berkowitz et al., 1983). When the particle hits the wall, it is reflected elastically. The radius R_{nuc} of the spherical wall is set to be R_{nuc} = 1.272 μm and 0.856 μm, obtained from the observed nuclear volumes for the log and stationary

phases, respectively. For each nucleus size, 1000 trajectories each with 2^{16} steps were generated and their statistical average was taken to calculate MSD."

Comment 8:
Figs. 5D, E and F: Since the cell length, the sizes of nuclear area and those of chromosome area are larger in cdc2-L7 mutant cells growing in log phase at 32 C than those in the wild-type cells (page 15 lines 14-15), significant increase of them in the mutant cells in stationary phase does not necessarily indicate that the CDK activity is required for changes in these size and length upon the entry into stationary phase. Although these sizes are significantly larger in the mutants than in the wild-type cells in stationary phase, they are obviously smaller in the mutant cells in stationary phase than those in log phase, indicating reduction of these sizes occur upon the entry into stationary phase even in the absence of wild-type CDK. The authors should calculate the ratio of these sizes between log phase and stationary phase cells, and compare these ratio between the wild-type and the mutant cells.

Our response:
We have included the cell volumes and the nuclear to cell volume ratio (N/C ratio) and discussed the regulation of the nuclear size in stationary phase in the revised version. The N/C ratio is constant irrespective of cell size in cycling cells (Neumann and Nurse, 2007). In addition, it was very recently reported that the nuclear size is determined by osmotic forces (Lemierre et al, 2022). In the osmotic force-dependent nuclear size regulation, the ratio of the numbers of osmotically active molecules in the nucleus and the whole cell determines the nuclear size. We found that the N/C ratio significantly reduced in stationary phase and GD cells, and despite the differences in the nuclear sizes, the N/C ratio was not significantly different between stationary phase and GD cells. This indicates that the intracellular state that governs the osmotic forces in stationary phase and GD cells are different from that in log phase cells and that the intracellular states are similar in stationary phase and GD cells.

Furthermore, despite the differences in nuclear sizes, the N/C ratios were not significantly different between *cdc2-L7* and wild-type cells in log phase at 32°C. This suggests that the observed nuclear size increase in *cdc2-L7* cells results from the larger cell size and not from impaired nuclear size reduction. However, it is apparent that proper cell size regulation in log phase is crucial for the subsequent stationary phase state. Moreover, there is no doubt that CDK reduces cell size by regulating the preceding cell cycle. Therefore, these results support our conclusion that CDK-dependent regulation of the preceding cell cycle is crucial for the establishment of proper stationary phase state. Based on these results, we have discussed how the osmotic forces change in stationary phase and GD cells and how CDK contributes to the stationary phase regulation.

Along these changes, we described methods for cell and nuclear size determination. Changes in description are following.

Line 10 on page 7
"Furthermore, the nuclear to cell volume ratio (N/C ratio), which has been reported to be constant irrespective of cell size (Neumann and Nurse, 2007), was significantly smaller (Fig. 1E; Fig. S1G). Thus, the nuclear size reduction observed in stationary phase cells did not originate simply from the reduction in cell size."

Line 3 on page 8
"The N/C ratio was also reduced but not significantly different from that of stationary phase cells (Fig. 1E; Fig S1G), suggesting that the observed nuclear size differences from stationary phase originate from the cell size differences."

Line 21 on page 13
"Sizes of the cell, the nucleus, and the chromosome-occupying space decreased, but were significantly larger compared with wild-type cells (Fig. 5D-F; Fig. S4 and S5A). These differences were already evident in log phase and despite the larger cell and nuclear sizes, there was no significant difference in N/C ratio between log and stationary phases (Fig. 5G), suggesting that the larger nuclear size result from the larger cell size."

Line 19 on page 14

“However, the N/C ratio was not significantly different (Fig. 5G), suggesting that the larger nuclear size in cdc2-L7 cells results from their larger cell size.”

Line 3 on page 21
“In *cdc2-L7* cells, the differences were already evident in log phase, and nuclear and chromosome size reduction occurred in stationary phase. These observations suggest that the observed improper stationary phase state originates from the improper state of cycling cells and not from impairment in stationary phase-associated changes. Even if so, it is obvious that CDK-dependent regulation of the preceding cell cycle is crucial for a proper stationary phase state. It is also apparent that CDK induces cell size reduction by modulating the preceding cell cycle (Kelkar and Martin, 2015; Petersen and Nurse, 2007; Yanagida et al., 2011). Furthermore, Cdc2 activity is required for Cdc2 nucleolar accumulation and nuclear size reduction upon acute glucose depletion. All these results support the idea that CDK plays a crucial role in stationary phase establishment before and upon stationary phase entry. Our viability analyses of stationary phase and GD cells also provided evidence to support this hypothesis (Fig. 8A -E).”

Line 15 on page 21
“Nuclear size is determined by osmotic force balance, which depends on the ratio of the numbers of osmotically active molecules in the nucleoplasm and the cytoplasm (Lemiere et al., 2022). The osmotic force balance is constant irrespective of cell size in cycling cells (Lemiere et al., 2022), while it is markedly changed in stationary phase and GD cells, as shown by a reduction in the N/C ratio. Furthermore, solidification of the cytoplasm occurs in stationary phase and GD cells (Heimlicher et al., 2019; Joyner et al., 2016), which likely increases the osmotic pressure on the nucleus if the gelation is induced by increased hydrophilicity, expressed as a decrease in the Flory-Huggins parameter x (Strobl, 2007). Therefore, a reduction in the intranuclear molecules and/or cytoplasmic solidification are likely to cause nuclear size reduction. In addition, since lipid metabolism and membrane flow contribute to nuclear size regulation (Kume et al., 2017; Kume et al., 2019), alteration in the nuclear membrane homeostasis may further contribute to the nuclear size reduction. CDK may induce nuclear size reduction by regulating nuclear transport, cytoplasmic solidification, and/or nuclear membrane homeostasis.”

Line 3 on page 30
“Cell volume was calculated using the Pombe Measurer ImageJ plugin (http://www.columbia.edu/~zz2181/Pombe Measurer.html) (Pino et al., 2021).”

Line 6 on page 30
“The nuclear volume was determined by regarding the nucleus as a spheroid with the semi-axis lengths, a, b, and $(a + b)/2$, where a and b are the lengths of the semi-axes of the fitted ellipse.”

Reviewer 2 Comments for the Author:
1. Statistics analyses are mostly well described. However, the presentation is yet inadequate on some points. In Fig8A-D, there is no explanation for statistics. If the description of statistics for Fig8E is also applied to these data, the authors should use some multiple comparison method because they would repeatedly test in multiple time points. If the authors would like to focus on the specific time point, they should explain the rationale. Instead, the authors might use the
other statistical test, which can directly compare the viability curve, such as log-rank test, rather than using multiple t-test.

Our response:
Viability data obtained by the microneedle dissection varied and sometimes were larger than those at the previous data points due probably to small sample numbers. The log-rank test assumes that viability reduces over time and cannot be applicable to our datasets in a regular manner. To apply the log-rank test, the data at the previous time points were used when the data were larger, and the P values obtained by the log-rank test are shown in Fig. 8. However, to avoid incorrect statistical evaluation by this non-formal log-rank test, Student’s t-test of datasets at the last time points were not removed in the revised version. This is written in the legend of Fig. 8 as follows.

Line 14 on page 42

"*P* values were obtained using the log-rank test, in which the viability data at the previous time points were used when the data were larger than the previous data due to statistical fluctuations. To avoid incorrect statistical evaluation with the non-formal log-rank test, the unpaired, two-tailed Student's t- test was also used to analyze the data sets at the last time points (*P<0.05; **P<0.0005; ns: not significant)."

2. The amount of CDK in the nucleolar should be quantified in Fig7 because the localization of CDK in the nucleus and nucleoli does not seem mutually exclusive, but rather there is some residual nucleoli localization with analog and residual nuclear localization in control as well. Some quantitative characteristics, such as nucleoli signal intensity normalized by that of the the nucleus should be useful to compare the localization changes in addition to the categorical classification in Fig7B.

Our response:
Following the reviewer's suggestion, we quantified intensities of Cdc2 signal in the nucleolus and the chromosomal region and showed the signal intensity of Cdc2 in the nucleolar region relative to that in the chromosomal region in Fig. 7C. We also quantified the nucleolar signal of Cdc2 variants shown in Fig. 6 and showed results in Fig. S6A-D. Although the relative nucleolar signal intensities were underestimated due to overlapping of the nucleolar region with the chromosomal region in our quantification method, the results are consistent with our qualitative observations and further confirm our conclusion. Changes in description are following.

Line 3 on page 16
"Signal intensities of Cdc2 in the nucleolar region relative to those in the chromosomal region confirmed these localization patterns (Fig. S6A-E)."

Line 13 on page 30
"Relative intensity of Cdc2 signal in the nucleolar region to that in the chromosomal region was determined as follows. Each deconvolved image set was combined to form a quantitative projection using an additive image projecting method. Then, the sums of the Cdc2 signal intensities in regions of the nucleus and chromosomes were obtained by subtracting the background signals. The total signal intensity in the nucleolar region was obtained by subtracting the total chromosomal signal intensity from the total nuclear intensity. The relative intensity of the nucleolar signal was determined using the obtained nucleolar and chromosomal signal intensities per area. Note that the relative nucleolar signal intensity was underestimated in this analysis since the nucleolar region partially overlapped with the chromosomal region in the two-dimensional projection due to the shape and the positioning of the nucleolus."

3. I wondered why cdc2-L7 phenotypes in the stationary phase were already apparent in the log phase cell (Fig5D-G). The authors need to supply rational excuses for this phenomenon. I guess that cell length increment and nuclear expansion in log phase cdc2-ts cell might be explained by just the delay in the cell cycle progression. But, I am not sure why the chromosome area, mobility, and separation were affected by CDK inactivation in the log phase.

Our response:
The nuclear size increase seen in *cdc2-L7* cells probably resulted from the larger cell size (please see our response to the comment 8 by the reviewer 1). On the other hand, we think it likely that CDK regulates chromosome compaction, chromosome flexibility, and sister chromatid association in log phase since CDK regulates histone modification and activities of condensin and cohesin, and that a reduction in CDK activity leads to the chromosomal changes seen in *cdc2-L7* cells. It is also possible that the chromosome compaction level is coupled with the nuclear size by an unknown molecular mechanism and that the chromosomal changes seen in *cdc2-L7* cells resulted from the larger nuclear size. These possibilities are described in the Discussion section. Along these changes, we simplified discussion about CDK-dependent regulation of chromosome fluctuation. Changes are following.

Line 9 on page 22
"..., which likely represses chromosome fluctuation."

Line 17 on page 22
“…and repress chromosome fluctuation ...”

Line 18 on page 22
“CDK may similarly regulate chromosome compaction and fluctuation in cycling cells, accounting for the increase in chromosome size and fluctuation seen in cycling *cdc2-L7* cells. We also cannot exclude the possibility that the chromosome compaction level is coupled with the nuclear size by an unknown molecular mechanism and that CDK changes the chromosome compaction and fluctuation by regulating nuclear size.”

Line 7 on page 23
“In *cdc2-L7* cells, sister chromatid separation may be increased at an undetected level during log phase, resulting in an increase in sister chromatid separation in stationary phase.”

4. The authors performed conditional CDK inactivation before and after the establishment of the stationary phase in Fig8C to distinguish whether CDK is required for either the establishment or maintenance of the stationary phase. The result apparently suggested that CDK requires sole for establishment. However, I have a concern regarding the discrepancy between Fig8B and Fig8C in respect of the phenotype severity. If CDK is required only for the establishment, the phenotype in Fig8C and Fig8B should be comparable, but conditional knockdown before the establishment of the stationary phase does not affect so much. The authors should explain in the manuscript why the effect of CDK inhibition before the stationary phase is subtle.

Our response:
At 32 °C, viability of wild-type cells more rapidly reduced in stationary phase than at 25 °C. The more prominent loss of cell viability in the continuous 32 °C incubation probably reflects additive effects of cdc2-independent viability reduction at 32°C in stationary phase. This is described in the revised version as follows.

Line 10 on page 17
“However, when temperature was downshifted after stationary phase entry, a less severe reduction in viability was observed (Fig. 8B and C). This probably reflects the lack of cdc2-independent viability reduction at 32 °C (13 days after stationary phase entry, the mean cell viability of wild-type cells was 93.3 ± 2.3% at 25°C but 67.0 ± 7.3% at 32°C).”

Minor suggestions:
1. On page 15, line 13, "The separation frequencies of ~". The authors clarify the comparison in this sentence, like "The separation frequency of cdc2-L7 cells increased compared to WT".

Our response:
We changed the description as follows.

Line 9 on page 14
“The separation frequencies of sister chromatid loci in cdc2-L7 cells significantly increased in stationary phase compared with wild-type cells (Fig 5I).”

2. Too low resolution Figure S5.

Our response:
We increased the resolution of Figure S5 (Fig. S6F and G in the revised version).

Other changes
(1) The role of Cdc2 nucleolar accumulation
We also changed discussion on the role of nucleolar Cdc2 accumulation. We noticed that Cdc28, a budding yeast CDK, was reported to be co-localized with ribonucleoprotein-containing cytoplasmic granules in stationary phase. Since the nucleolus also consists of ribonucleoproteins, colocalization of CDK with ribonucleoproteins is probably common among eukaryotes. This possibility is stated in the revised version as follows.

Line 4 on page 19
“On the other hand, in budding yeast, Cdc28, a sole CDK in this organism, is colocalized with ribonucleoprotein-containing cytoplasmic granules during stationary phase (Shah et al., 2014). These results suggest that CDK becomes commonly colocalized with the ribonucleoproteins in response to stress. Perhaps, this colocalization is crucial for the regulation of RNA metabolism.”

(2) Viability analysis of stationary phase and GD cells
In addition to the above changes, we removed viability results of co-cultivated stationary phase and GD cells in the revised version. We have very recently found that stationary phase culture medium contains an unknown compound(s), which is toxic to log phase cells and are currently trying to identify the compound(s). Therefore, we cannot exclude the possibility that when co-cultivated, the log phase cells lose viability more rapidly than stationary phase cells due to the unknown toxic compound(s) secreted by the stationary phase cells. Considering our unpublished new results, we decided to remove Fig. 4D. Accordingly, we removed the paragraph describing Fig. 4D (line 2 on page 14 in the old version) and stated the possible reason for the shorter survival period of GD cells as follows. We believe that removal of Fig. 4D does not affect our conclusion.

Line 11 on page 13
“… and suggest that GD cells are less resistant to glucose-depleted environments or more rapidly consume essential nutrients compared with stationary phase cells.”

Second decision letter

MS ID#: JOCES/2022/260727

MS TITLE: CDK actively contributes to establishment of the stationary phase state in fission yeast.

AUTHORS: Motoaki Hiraoka, Yuki Kiyota, Shinnosuke Kawai, Yusuke Notsu, Kohei Yamada, Katsuyuki Kurashima, Jing-Wen Chang, Shunsuke Shimazaki, and Ayumu Yamamoto
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

In the study by Hiraoka et al, the authors characterize fission yeast cells in stationary phase and those subjected to acute glucose depletion (GD). They find that, when the cells enter stationary phase, the sizes of the nuclei and chromosome-occupying space decrease, frequency of sister chromatid separation is reduced, and Brownian-like fluctuation of chromosomes are repressed. These changes are less evident in GD cells, and require fully-functional CDK before/upon the entry into stationary phase. When the cells enter stationary phase, intracellular localization of CDK changes dependent on its kinase activity. Concomitantly, chronological life span of the stationary phase cells is significantly longer than GD cells, and this extension of life span requires fully-functional CDK. These observations suggest a new role of CDK in log phase, which is required to change physiological state in subsequent stationary phase and important for cell survival.

Comments for the author

All the comments to the original version are appropriately responded in the revised manuscript. The added results and modifications further strengthen the manuscript and clarify the clams by the authors. This reviewer believes that this manuscript is now suitable for publication in Journal of cell science.

Reviewer 2

Advance summary and potential significance to field

The revised manuscript is much clearer and more accessible for readers to understand the role of CDK activity in the establishment of the stationary phase.
The additional data make their argument more convincing so that the current manuscript now provides useful insights into the mechanism underlying the stationary phase establishment for future studies.

Comments for the author

All my concerns have been clarified, and I have no more requirements about the current revised manuscript.