



# CDKD-dependent activation of CKDA;1 controls microtubule dynamics and cytokinesis during meiosis

Kostika Sofroni, Hirotomo Takatsuka, Chao Yang, Nico Dissmeyer, Shinichiro Komaki, Yuki Hamamura, Lev Böttger, Masaaki Umeda, and Arp Schnittger

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*Scientific Editor: Marie Anne O'Donnell*

## Transaction Report:

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

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August 15, 2019

Re: JCB manuscript #201907016

Prof. Arp Schnittger  
University of Hamburg  
Department of Developmental Biology  
Ohnhorststr. 18  
Hamburg, Hamburg 22609  
Germany

Dear Prof. Schnittger,

Thank you for submitting your manuscript entitled "Conversion of synchronous into successive cytokinesis during meiosis of Arabidopsis". The manuscript has been evaluated by expert reviewers, whose reports are appended below. Unfortunately, after an assessment of the reviewer feedback, our editorial decision is against publication in JCB.

You will see that the reviewers appreciate the study comprises a detailed characterization of the meiosis phenotype of CDKD mutant Arabidopsis. As Reviewer #1 notes, prior work has indicated that CDKs play a role in microtubule dynamics during mitosis, so it is not too surprising that these enzymes and their regulators also have an impact during meiosis. We had noted during our initial evaluation of the manuscript that the substrates for CDKD / CDKs responsible for the phenotypes described had not been identified. Reviewer #1 also emphasizes this issue, therefore, we feel it would be necessary to provide some additional insight to broaden the overall advance for JCB. As this may require identifying targets for these kinases that affect microtubule dynamics, or some other process that determines synchronous versus successive cytokinesis, we expect that it would take considerably longer than our standard revision period.

Although your manuscript is intriguing, I feel that the points raised by the reviewers are more substantial than can be addressed in a typical revision period. If you wish to expedite publication of the current data, it may be best to pursue publication at another journal.

Given interest in the topic, I would be open to resubmission to JCB of a significantly revised and extended manuscript that fully addresses the reviewers' concerns and is subject to further peer-review. If you would like to resubmit this work to JCB, please contact the journal office to discuss an appeal of this decision or you may submit an appeal directly through our manuscript submission system. Please note that priority and novelty would be reassessed at resubmission.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss the reviewer comments further once you've had a chance to consider the points raised in this letter. You can contact the journal office with any questions, [cellbio@rockefeller.edu](mailto:cellbio@rockefeller.edu).

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Arshad Desai, Ph.D.  
Editor

Marie Anne O'Donnell, Ph.D.  
Scientific Editor

Journal of Cell Biology

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

Comments

The manuscript by Sofroni and co-authors addresses the function of CDKs in development of pollen grains in Arabidopsis. The main claim of this work is that by altering activity of CDK, the morphology of meiotic microtubule arrays is affected and the spatial progression of cytokinesis is altered. The key strength of this work is very detailed characterization of the mutant phenotypes. It is clear that a lot of efforts was invested in the recording and analysing videos of meiotic cells. The conclusion about the role of CDK in organization of microtubules during meiosis and overall microsporogenesis is justified. However, there are several weaknesses.

1. The role of CDKs in microtubule organization had been shown in several studies. CDKs were localized to the pre-prophase band in stomata and roots in maize (Colasanti et al., 1993), application of CDK inhibitors resulted in the loss of spindle polarity (Binarova et al., 1998). Later on, a detailed study by Weingartner and colleagues (Weingartner et al., 2001) provided localization data of CDKs during cell cycle and demonstrated localization of the kinase on all microtubule arrays. Expression of non-degradable cyclin B1, which would result in constitutive active CDKs, disrupted phragmoplast organization and abrogated cytokinesis (Weingartner et al., 2004). Considering conservation of mechanisms that regulate microtubule dynamics through plant development, it is not surprising that CDKs also regulate microtubule dynamics in meiosis.

2. Quantification of the mutant phenotypes is very comprehensive, but provides limited insights into how the kinase activity controls the balance between the synchronous and successive cytokinesis. Changing activity of a protein kinase may cause pleiotropic phenotypes by altering other signalling pathways. Both CDKA;1 and CDKd:3 are expressed in the tapetum cells and at least some of the observed phenotypes could non cell-autonomous. This should be tested. It is essential to answer the question whether effects on microtubules, transition through the meiotic stages, and cytokinesis are governed through phosphorylation of the same or different proteins.

3. The design of experiments presented in Figure 6 lacks appropriate positive and negative controls, which should be a known microtubule-binding protein and a known cytoplasmic protein. Ideally, it should be a CDKA1 without the microtubule-targeting domain. This result is not discussed either. Considering published data on CDK in mitosis (Weingartner et al., 2001) CDK1A;1 does not seemingly bind microtubules. The Pearson's co-localization coefficient is not appropriate in these experiments as both tubulin and CDKA;1 exhibit considerable cytoplasmic signal.

4. On page 10 the authors write "we also found ectopic spindle/phragmoplast-like structures" and refer to Figure S5G. However, Figure S5G lacks any apparent "ectopic" spindles or phragmoplasts.

Instead, microtubules form a large aggregate. This aggregate could be a bundle, which by the way are seen in almost every tapetum cell.

5. The quality of microtubule images is poor. For this reason it was not possible to examine how specific are alterations in the morphology of the microtubule arrays. In most figures, they appear misshaped, which could be a non-specific effect. It is important that authors provide a better resolution images.

## References

- Binarova, P., J. Dolezel, P. Draber, E. Heberle-Bors, M. Strnad, and L. Bogre. 1998. Treatment of *Vicia faba* root tip cells with specific inhibitors to cyclin-dependent kinases leads to abnormal spindle formation. *Plant J.* 16:697-707.
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## Reviewer #2 (Comments to the Authors (Required)):

This is an interesting story. The major conclusion of the study is that a reduction in CDKA;1 activity results in a shift from synchronous to successive cytokinesis during male meiosis. This is interesting from both evolutionary and functional perspectives as synchronous cytokinesis (one cytokinesis giving rise to four gametes) occurs in the model organism *Arabidopsis* whereas successive cytokinesis (with a cell division after meiosis I and meiosis II) occurs in some crop species such as maize. CDKA;1 activity was slightly reduced in a previously characterized mutant CDKA;1T14V;Y15F (referred to as VF) and more substantially reduced in a new mutant CDKA;1T14V;Y15F;Thr161D (referred to as VFD). The VF mutant variant was combined with multiple CDKD alleles in which CDKD function was reduced in a step-wise fashion. This yielded a synthetically enhanced phenotype, suggesting that CDKA;1 regulates meiosis in a CDKD-dependent fashion.

The conclusions drawn by the authors are overall supported by the datasets but there are two major concerns:

1. The phenotypes are difficult to see in the main text (which I attempted to read as a print out, to no avail) and are only apparent in the movies or when one zooms in to the original figures. It would overall really help if the authors presented for the main close ups of single defects at representative stages (univalents at metaphase I, lagging chromosomes in interkinesis, chromosome bridges in telophase II) rather than an overview with very small panels representing each stage. Specific examples for poor documentation of phenotypes in the main are outlined below.

a. The authors conclude that CDKDs co-localize with CDKA;1 in the nucleus.

Here an issue is that the CDKA;1mTurquoise2 construct yields a very diffuse pleiotropic signal with

variable degrees of enrichment in the nucleus. A considerable moiety of the signal does not overlap and this should be quantified. The text here is misleading w.r.t. CDKA;1 localization and co-localization. Quantification of how much of the total CDKA;1 signal is in the nucleus and a neutral description of its localization patterns throughout meiosis (as presented later in the results) are required at this stage in the results. Also, the sentence stating that the high level of co-localization is consistent with an in vivo interaction is too tendentious for the results and should be moved to the discussion.

b. The authors conclude that double mutants in CDKD genes have severe meiotic defects including a decrease in the number of crossovers and unbalanced chromosome pools. I am sure this is correct but I need to take the author's view on this. The current figure 2 is very nice at providing an overview but as a reviewer I would need in lieu of panel A close ups with wild-type and a representative mutant to show the above-mentioned deviations from the wild type, followed by the graphs in B. The current figure 2 A could then be relegated to the supplement.

c. Premature cell wall formation in interkinesis is difficult to see as a negative stain in a chromosome spread (Figure 3B), or in bright field images (Figure 5J and 5K). This should be visualized directly by a cross wall/cell wall stain such as callose. In two of the supplementary movies, cross wall formation is more clear and it would help to have a closer snap shot of the relevant stage for the main.

d. The rendition of microtubule dynamics would benefit from a greater zoom, higher resolution and deconvolution.

2. The discussion of synergy (or synthetic enhancement) is flawed. First, the authors need to adequately document via quantification the observation that the phenotypes are synergistic and not additive. For this, Fig. 3C would need to show bars for WT (present), VF alone (present), *cdkd;3/-* (missing in Fig. 3, partially shown and close to WT in Fig. 2B but not shown for all parameters) and then the combined mutants (shown). It would then help if the authors could place a dotted horizontal line where an additive phenotype would be expected to make it clear that what is seen is considerably stronger than an additive phenotype.

Second, while it can certainly be concluded from a synergistic double mutant phenotype that CDKA;1 and CDKD;3 act in concert, alternative interpretations such as parallel pathways are also possible. Thus, the introduction to this results section (at the bottom of p. 7), which reads that synergy would be a way of showing that the gene products are in the same pathway needs to be deleted and the approach worded more openly. On its own, synergy demonstrates a functional link, but cannot be overinterpreted. The wording should really be toned down accordingly.

Minor comments:

3. Please be sure to distinguish between arrows and arrowheads in the figure legends.

4. The movies should all have boxes and arrows or other tools to highlight what the reader should be focusing on (currently some do and some don't).

**Detailed response to the reviewer comments**

**Reviewer #1**

**The manuscript by Sofroni and co-authors addresses the function of CDKs in development of pollen grains in Arabidopsis. The main claim of this work is that by altering activity of CDK, the morphology of meiotic microtubule arrays is affected and the spatial progression of cytokinesis is altered. The key strength of this work is very detailed characterization of the mutant phenotypes. It is clear that a lot of efforts was invested in the recording and analysing videos of meiotic cells. The conclusion about the role of CDK in organization of microtubules during meiosis and overall microsporogenesis is justified. However, there are several weaknesses.**

We are happy about the constructive feedback and would like to thank this reviewer for his/her comments and the time invested reviewing our manuscript. To emphasize what the reviewer regarded as the strong point, we have also adjusted our title and say now: “CDKD-dependent activation of CKDA;1 controls microtubule dynamics and cytokinesis during meiosis of Arabidopsis”. We hope that this is in the line of the reviewer’s thoughts.

**1. The role of CDKs in microtubule organization had been shown in several studies. CDKs were localized to the pre-prophase band in stomata and roots in maize (Colasanti et al., 1993), application of CDK inhibitors resulted in the loss of spindle polarity (Binarova et al., 1998). Later on, a detailed study by Weingartner and colleagues (Weingartner et al., 2001) provided localization data of CDKs during cell cycle and demonstrated localization of the kinase on all microtubule arrays. Expression of non-degradable cyclin B1, which would result in constitutive active CDKs, disrupted phragmoplast organization and abrogated cytokinesis (Weingartner et al., 2004). Considering conservation of mechanisms that regulate microtubule dynamics through plant development, it is not surprising that CDKs also regulate microtubule dynamics in meiosis.**

We agree that CDKA;1 has been implicated to regulate microtubules in mitosis by localization studies. We also apologize if we have raised the impression that we have described here for the first time microtubule defects related to CDK activity, this was not our intention and we have double checked that the work, referred to by the reviewer is cited in the revised manuscript.

However, we also like to kindly remark here that *cdk* mutants in plants, at least to our knowledge, have not been analyzed with respect to their effects on microtubules. In Arabidopsis, there are 12 CDK genes and many more CDK-like genes, most of them only poorly characterized, of which most if not all could be targeted by CDK inhibitors. Conversely, many of these CDK and CDK-like genes could probably be activated by overexpression of a cyclin based on interactome data. Thus, we think that it is not a trivial deduction that CDKA;1 controls microtubules in meiosis especially since microtubule structures in meiosis are strikingly different from the ones found in mitosis, e.g. there is no pre-prophase band in meiosis and cytokinesis, and with that the phragmoplast, follows a for plants very unusual pattern from the outside into the cell. We would also not have guessed before we started these experiments that, for instance, the microtubule cage around the nucleus in prophase I of meiosis disappears in a CDKA;1-CDKD dosage dependent manner as we show in our manuscript.

None-the-less, we agree that for a publication in JCB considerable advance has to be presented. Hence, we have substantially expanded our work and include in this revised version the analysis of CYCB3;1, which we show forms a complex with CDKA;1. Our work includes analysis and live cell imaging of microtubules in *cycb3;1*, double mutant analysis with the hypomorphic *cdka;1* mutants, localization and co-localization studies as well as a newly developed assay to apply oryzalin to meiocytes. These added data unambiguously reveal that CYCB3;1 is an important regulator of microtubules in meiosis.

These new data are presented in the new figure 8, 9, and S5, as well as in Video 7, 8, 9 and 10. With this, we hope that the reviewer agrees that this addition represent a significant advance in the functional analysis of microtubule regulation during meiosis.

**2. Quantification of the mutant phenotypes is very comprehensive, but provides**

**limited insights into how the kinase activity controls the balance between the synchronous and successive cytokinesis. Changing activity of a protein kinase may cause pleiotropic phenotypes by altering other signalling pathways. Both CDKA;1 and CDKd;3 are expressed in the tapetum cells and at least some of the observed phenotypes could non cell-autonomous. This should be tested. It is essential to answer the question whether effects on microtubules, transition through the meiotic stages, and cytokinesis are governed through phosphorylation of the same or different proteins.**

The reviewer raises two interesting questions here. First, whether CDKA and CDKD function in a cell-autonomous manner and second, what the phospho-targets of CDKA;1 are in meiosis.

Concerning the cell autonomy, the reviewer is right that several mutants, especially in small RNA pathways, in which tapetum formation and/or differentiation is affected also show defects in meiosis. We find that CDKA;1 and CDKDs, as pointed out by the reviewer, are expressed in both the tapetum cells and in meiocytes. Furthermore, there are no obvious defects in the tapetum layer in mutants with reduced CDKA;1 activity levels. Therefore, there is no indication that the effect we see comes from a non-cell-autonomous and/or indirect function of CDKA;1 and CDKDs in tapetum cells. Moreover, mutants with defective tapetum have so far not been found to affect chromosome dynamics in meiosis as we see here, e.g. the appearance of chromosome bridges or univalents. Consistent with that, we have recently shown that chromosome dynamics in meiosis is independent from tapetum development (Prusicki et al. eLife 2019). Our newly added data on CYCB3;1 also supports a cell-autonomous nature of the phenotypes reported here since CYCB3;1 is present in meiocytes throughout prophase I and localizes to the spindles where defects can be seen together with a reduction of CDKA;1 activity. CYCB3;1 is occasionally seen in very few tapetum cells, likely at the very end of mitosis, but it is difficult to explain how a defect in one or two cells could affect the spindles in all meiocytes of one anther. Thus, given that cell-autonomy is very difficult to test in reproductive tissues, especially in meiosis due to the lack of very tight and specific promoters, we hope that it is sufficient that we have added a discussion point on the question of cell autonomy.



With the next question, the reviewer is basically asking for comparative phospho-proteomic studies in which we compare wildtype with mutant meiocytes of *Arabidopsis* since the substrates of Cdks with respect to microtubule organization are not known in plants. Such an experiment has to our knowledge not been done in any plant so far. In a major effort, the lab of Chris Franklin has obtained a phospho-proteome of the much larger species *Brassica napus*, which also has larger meiocytes. However, this work was “only” done in the wildtype. We also like to stress that even if such differences in phosphorylated proteins could be detected it does not mean that they are biologically relevant and many experiments have to follow including the mutation of the phosphorylated sites in these target proteins and their functional test in the respective mutant backgrounds. Please note that even in yeast, probably the most advanced model system to study cell division, the biologically relevant phosphorylation sites that control proteins involved in the spindle and other microtubule organization are not or barely known to our understanding.

Thus, while such a comparative phospho-proteome is a highly interesting data set, it represents a research plan for several years and we hope that the reviewer agrees that such an experiment goes much beyond the scope of this work (please also note that we have fully exhausted the number of main and supplementary figures as well as videos). None-the-less, we have added localization studies of MAP65-3 (Figure 6), which has several predicted CDKA;1 phosphorylation site. This work revealed that MAP65-3 is not as distinct localized in plants with reduced CDKA;1 activity. We can currently not determined whether this altered localization pattern is due to lower levels of MAP65-3 phosphorylation but we have added a discussion point on CDKA;1 substrates including MAP65. We hope that this gives the readers some inspiration for future experiments.

**3. The design of experiments presented in Figure 6 lacks appropriate positive and negative controls, which should be a known microtubule-binding protein and a known cytoplasmic protein. Ideally, it should be a CDKA1 without the microtubule-targeting domain. This result is not discussed either. Considering published data on CDK in mitosis (Weingartner et al., 2001) CDKA;1 does not seemingly bind microtubules. The Pearson's co-localization coefficient is not**

**appropriate in these experiments as both tubulin and CDKA;1 exhibit considerable cytoplasmic signal.**

We thank the reviewer for bringing up this important point. We have now included a new figure 7 (based on the old figure 6) a negative control in panel A: free mVenus expressed from the CDKA;1 promoter in combination with tubulin fused to RFP (PRO<sub>CDKA;1</sub>:mVenus x PRO<sub>RPS5</sub>:TagRFP:TUA5). This control does not show an enrichment on the spindles in metaphase I nor metaphase II or other microtubule structures. Thus, we conclude that CDKA;1 is indeed localized to the spindles and other microtubule structures as shown in panel B.

It is likely that the microtubule localization of CDKA;1 is mediated by its cyclin partner. As pointed out in our preceding answer, we have identified CYCB3;1 as one out of likely several cyclins that mediate this accumulation pattern.

We also agree with the reviewer that the Pearson's co-localization coefficient is not appropriate and instead, we provide in this figure the intensity plot profiles from one line passing through the middle of the nucleus at late prophase and containing the section of first and second spindles during metaphase I and metaphase II, respectively.

**4. On page 10 the authors write "we also found ectopic spindle/phragmoplast-like structures" and refer to Figure S5G. However, Figure S5G lacks any apparent "ectopic" spindles of phragmoplasts. Instead, microtubules form a large aggregate. This aggregate could be a bundle, which by the way are seen in almost every tapetum cell.**

We agree that the characterization of these microtubule structures was not very detailed and we have now followed up this comment of the reviewer by introgressing and subsequently imaging a well-known marker of microtubule dynamics, MAP65-3, in our mutants. We find that MAP65-3 is prematurely expressed at late prophase in plants with low CDK levels (Figure 6E-I and Video 6). Based on the presence of this marker, we also think that it is justified to refer to these structures as phragmoplast-like structures since they are not a random aggregate of microtubules but follow

indeed a pattern that resembles early phragmoplasts. Please also note that MAP65-3 does not mark any microtubule bundles seen in tapetum cells.

**5. The quality of microtubule images is poor. For this reason it was not possible to examine how specific are alterations in the morphology of the microtubule arrays. In most figures, they appear misshaped, which could be a non-specific effect. It is important that authors provide a better resolution images.**

Most of our pictures were derived from movies, which typically do not have the highest spatial resolution due to the need to preserve the fluorophore for a long time. These movies were very instrumental for us to identify these defects in the first case. However, we agree with the reviewer that it was often difficult to see the defects of the mutants presented. We now provide details of microtubule arrays with better quality in the new figure 4. These images clearly show how low levels of CDKs affect microtubule patterns during meiosis. We additionally quantified these defects, for instance in Figure 4J and show that they are often dosage dependent providing additional support that these defects have a clearly defined genetics base.

#### **Reviewer #2**

**This is an interesting story. The major conclusion of the study is that a reduction in CDKA;1 activity results in a shift from synchronous to successive cytokinesis during male meiosis. This is interesting from both evolutionary and functional perspectives as synchronous cytokinesis (one cytokinesis giving rise to four gametes) occurs in the model organism Arabidopsis whereas successive cytokinesis (with a cell division after meiosis I and meiosis II) occurs in some crop species such as maize. CDKA;1 activity was slightly reduced in a previously characterized mutant CDKA;1T14V;Y15F (referred to as VF) and more substantially reduced in a new mutant CDKA;1T14V;Y15F;Thr161D (referred to as VFD). The VF mutant variant was combined with multiple CDKD alleles in which CDKD function was reduced in a step-wise fashion. This yielded a synthetically enhanced phenotype, suggesting that CDKA;1 regulates meiosis in a CDKD-dependent fashion.**

**The conclusions drawn by the authors are overall supported by the datasets but there are two major concerns:**

We appreciate the positive and constructive feedback and we would also like to thank this reviewer for his/her comments.

**1. The phenotypes are difficult to see in the main text (which I attempted to read as a print out, to no avail) and are only apparent in the movies or when one zooms in to the original figures. It would overall really help if the authors presented for the main close ups of single defects at representative stages (univalents at metaphase I, lagging chromosomes in interkinesis, chromosome bridges in telophase II) rather than an overview with very small panels representing each stage. Specific examples for poor documentation of phenotypes in the main are outlined below.**

We agree with the reviewer that our figures were difficult to read. In the revised manuscript, we have taken this comment to our hearts and every movie has now be complemented by close-ups presented in corresponding figures. We hope that this helps the reader to see the reported defects clearly and to better understand our movies, especially given that the speed of the movies is sometimes fast. Additionally we have highlighted deviations from the wildtype by asterisks, arrows and circles in our figures.

For the chromosome spreads analyses, we added close ups of the defects in the new figure 1: univalents at metaphase I (1B), unbalanced pools in interkinesis and metaphase II (1C,D) and chromosome bridges in telophase II (1E). In addition, we focus only on those stages in which defects are visible.

**a. The authors conclude that CDKDs co-localize with CDKA;1 in the nucleus. Here an issue is that the CDKA;1mTurquoise2 construct yields a very diffuse pleiotropic signal with variable degrees of enrichment in the nucleus. A considerable moiety of the signal does not overlap and this should be quantified. The text here is misleading w.r.t. CDKA;1 localization and co-localization.**

**Quantification of how much of the total CDKA;1 signal is in the nucleus and a neutral description of its localization patterns throughout meiosis (as presented later in the results) are required at this stage in the results. Also, the sentence stating that the high level of co-localization is consistent with an in vivo interaction is too tendentious for the results and should be moved to the discussion.**

In the revised version of the manuscript we re-arranged the old figure 1, now figure S2, and provide a detailed quantification of the co-localization between CDKA;1 and CDKD;3 by a newly added co-localized pixel map and scatter plot analysis to substantiate our observations. The description of CDKA;1 localization in meiosis was previously published (Yang et al., 2020). As requested, we provide a detailed description of the accumulation pattern of all three CDKD through meiosis..

**b. The authors conclude that double mutants in CDKD genes have severe meiotic defects including a decrease in the number of crossovers and unbalanced chromosome pools. I am sure this is correct but I need to take the author's view on this. The current figure 2 is very nice at providing an overview but as a reviewer I would need in lieu of panel A close ups with wild-type and a representative mutant to show the above-mentioned deviations from the wild type, followed by the graphs in B. The current figure 2 A could then be relegated to the supplement.**

As already layout above, we have added close-up and commented panels (Figure 1B-E) of representative examples of meiotic defects: univalents, unbalanced pools in interkinesis and metaphase II, chromosome bridges in telophase II.

**c. Premature cell wall formation in interkinesis is difficult to see as a negative stain in a chromosome spread (Figure 3B), or in bright field images (Figure 5J and 5K). This should be visualized directly by a cross wall/cell wall stain such as callose. In two of the supplementary movies, cross wall formation is more clear and it would help to have a closer snap shot of the relevant stage for the main.**

We agree with the reviewer that this central point was not very well documented. Therefore, we have introgressed a plasma membrane marker (GFP:SYPI32) into our mutants and have filmed the dynamics of plasma membrane formation during cytokinesis. These data are presented in our new Video 4 and the corresponding high-resolution images in figure 5. Importantly, these data fully support our previous claims.

**d. The rendition of microtubule dynamics would benefit from a greater zoom, higher resolution and deconvolution.**

In this revised version, we have supplemented our live cell imaging of microtubule dynamics with higher-resolution and greater magnified images, please see for instance Figure 4. We think that the defects are indeed now easier to see than before. Similar to the above-described experiments, our conclusions have not changed when analyzing these more detailed pictures.

**2. The discussion of synergy (or synthetic enhancement) is flawed. First, the authors need to adequately document via quantification the observation that the phenotypes are synergistic and not additive. For this, Fig. 3C would need to show bars for WT (present), VF alone (present), *cdkd;3*<sup>-</sup> (missing in Fig. 3, partially shown and close to WT in Fig. 2B but not shown for all parameters) and then the combined mutants (shown). It would then help if the authors could place a dotted horizontal line where an additive phenotype would be expected to make it clear that what is seen is considerably stronger than an additive phenotype. Second, while it can certainly be concluded from a synergistic double mutant phenotype that *CDKA;1* and *CDKD;3* act in concert, alternative interpretations such as parallel pathways are also possible. Thus, the introduction to this results section (at the bottom of p. 7), which reads that synergy would be a way of showing that the gene products are in the same pathway needs to be deleted and the approach worded more openly. On its own, synergy demonstrates a functional link, but cannot be overinterpreted. The wording should really be**

**toned down accordingly.**

We agree with the reviewer that these genetic experiments can only give a clue but are not sufficient to draw conclusions about the mechanism of action. We have correspondingly down-tuned the introduction of this section. We also avoid the use of the word synergy since we feel that this could be misleading. None-the-less, we like to underline that the effects of the double mutant *VF cdka cdkd* are striking. There are hardly any defects in both single mutants (please see Figure 1F and Figure 2C). For instance, there are only 2% of meiocytes that have univalents in *VF cdka;1* and 0% of the meiocytes show univalents in *cdkd3;1/-*. Taking one functional allele of *CDKD3* away in a *VF cdka;1* mutant background results in 28% of meiocytes with univalents and having *cdkd3* in a homozygous state in *VF cdka;1* mutants causes the formation of univalents in 66% of the meiocytes analyzed. The situation is very similar for the other five phenotypic criteria applied. We initially added the suggested dotted line but removed it later again since we felt that this line is rather confusing (especially since the line was often not visible as it overlaid with the X-axis). If the reviewer still thinks that it is helpful, we are happy to add it again.

**Minor comments:**

**3. Please be sure to distinguish between arrows and arrowheads in the figure legends.**

We now use only arrows with different colors in the revised manuscript.

**4. The movies should all have boxes and arrows or other tools to highlight what the reader should be focusing on (currently some do and some don't).**

For all videos, we provide now close-ups in the main figures (except Video 1). The legend of the movies always refer to the respective figures containing the close-up for each time point. To further facilitate and guide the reader, we have added asterisks, arrows, and circles and suggested by the reviewer. With this we hope that

the presented mutant phenotypes are better understandable than before for a broad readership.



March 26, 2020

Re: JCB manuscript #201907016R-A

Prof. Arp Schnittger  
University of Hamburg  
Department of Developmental Biology  
Ohnhorststr. 18  
Hamburg, Hamburg 22609  
Germany

Dear Prof. Schnittger,

Thank you for submitting your revised manuscript entitled "CDKD-dependent activation of CKDA;1 controls microtubule dynamics and cytokinesis during meiosis". The manuscript has been seen by the original reviewers whose full comments are appended below. While the reviewers continue to be overall positive about the work in terms of its suitability for JCB, some important issues remain.

Both reviewers appreciate the significant revision effort and agree the manuscript is improved. While Rev #1 remains concerned about the level of novelty, Rev #2 and our editorial view is that this issue should not limit publication. Thus, we would like to invite you to address the remaining points related to the text and figures raised by the reviewers and return the manuscript to us for final consideration.

One point we would like you to consider when preparing the final revision is that the journal targets a broad cell biological audience. Thus, we would encourage use of nomenclature and labeling that is as accessible as possible, together with explication of system-specific features for a non-familiar reader. We encourage use of widely understood names (like "Cdk1") together with organism-specific gene names in superscript (or some analogous system) to help ensure that nomenclatural & system differences do not limit interest in and impact of the work.

Please also attend to the following formatting changes for resubmission:

- Provide the main and supplementary texts as separate, editable .doc or .docx files
- Provide main and supplementary figures as separate, editable files according to the instructions for authors on JCB's website paying particular attention to the guidelines for preparing images and blots at sufficient resolution for screening and production
- Provide tables as excel files
- Add paragraph after the Materials and Methods section briefly summarizing all "Online Supplementary Materials"

Our general policy is that papers are considered through only one revision cycle; however, given that the suggested changes are relatively minor we are open to one additional short round of revision. Please note that I will expect to make a final decision without additional reviewer input upon resubmission.

Please submit the final revision within one month, along with a cover letter that includes a point by point response to the remaining reviewer comments.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact me or the scientific editor listed below at the journal office with any questions, [cellbio@rockefeller.edu](mailto:cellbio@rockefeller.edu) or call (212) 327-8588.

Sincerely,

Arshad Desai, Ph.D.  
Editor

Marie Anne O'Donnell, Ph.D.  
Scientific Editor

Journal of Cell Biology

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

This is a revised manuscript by Sofroni et al. In response to my original review the authors added an extensive dataset demonstrating interaction between CDKA and Cyclin B, performed additional control experiments for the co-localization of CDKA and microtubules during metaphase I and metaphase II, provided an additional dataset to characterize the phragmoplast-like structures that form prior to NEB in prophase, added localization of the anti-parallel microtubule bundling factor MAP65-3 during meiosis. A significant number of figures and supplemental data were added, and corresponding textual changes done. I highly appreciate the efforts that authors invested in the revised manuscript.

Overall this paper contains a high-quality (and large) dataset. The volume of genetic analyses is very impressive. Clearly, it was a very laborious project. However, the JCB manuscripts are judged by novelty of the mechanistic insights into cellular processes and by their interest to the broad cell biology community. Identification of specific cyclin is highly appropriate for the storyline, but this finding is not surprising considering that Cyclins are known to function as CDK activators. I lack sufficient expertise on the protein phosphorylation during yeast or mammalian cell division to verify the claim in the rebuttal letter, but identification of a substrate would significantly advance our understanding of the signalling pathways during meiosis.

Without an idea about which pathways are affected in the mutants, some conclusions seem too speculative. For example, the physiological relevance of altered CDK activity in the context of transition from simultaneous to sequential cell division is questionable. Are species with sequential division have reduced CDK activity? Localization of MAP65-3 shows no discernible modifications in the mutant background and as far as I can judge from the figures, MAP65-3 is unlikely involved in the above change of the cell division.

Another essential issue remains association of CDK with microtubules during meiosis. I commented on the lack of appropriate negative and positive controls. In the revised manuscript mVenus driven by CDKA<sub>1</sub> promoter was used as a negative control and the data included in Figure 7A. The image

acquisition parameters in this experiment were apparently optimized for the neighbouring tapetum cells that have strong mVenus signal in the nucleus. For this reason cytoplasmic signal in the meiotic cells appears weak. Adjusting the levels in this image produced a similar fluorescence signal in the spindle region as shown for these stages in Figure 7B. Given that, I'm not convinced mVenus signal does not accumulate around spindle microtubules. Proving specific localization of proteins on the spindle, unless there is direct association with microtubules, is technically challenging as this cytoplasm-rich region naturally accumulates almost every cytoplasmic protein. A strongly expressing control is necessary. Also measuring association of the CDKA;1 with spindle microtubules versus a strongly-expressed control would strengthen this conclusion.

Following from the above, proving association between mitotic spindle and CDKA;1 may not be necessary. As shown in Figure 6, the "phragmoplast-like structures" in the mutant appear already during pre-prophase before the NEB and spindle assembly. Hence, the observed defects in the microtubule dynamics and the sequential cytokinesis may not require association between the kinase and microtubules. The regulatory phosphorylation events could occur on cortical microtubules, on plasma membrane, or in the cytoplasm.

In this version the authors call microtubule bundles in the prophase the "phragmoplast-like structures" because they are labelled by MAP65-3. First, anaphase spindle midzone also accumulates MAP65-3. It means presence of this marker alone does not warrant defining these structures as the phragmoplasts, though I also find this rather tempting. Second, the phragmoplast is characterized by deposition of membrane to the midzone. The membrane visualization in Figure 5D shows membrane deposition only during cytokinesis. The most accurate would be to call these structures anti-parallel microtubule bundles. Furthermore, semantically "phragmoplast-like structure" sounds confusing as it implies cells skip metaphase and anaphase to proceed into cytokinesis while having intact nuclear envelope.

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

The major conclusion of this study is that a reduction in CDKA;1 activity results in a shift from synchronous to successive cytokinesis during male meiosis. This is interesting from both evolutionary and functional perspectives as synchronous cytokinesis (one cytokinesis giving rise to four gametes) occurs in the model organism *Arabidopsis* whereas successive cytokinesis (with a cell division after meiosis I and meiosis II) occurs in some crop species such as maize. CDKA;1 activity was slightly reduced in a previously characterized mutant CDKA;1T14V;Y15F (referred to as VF) and more substantially reduced in a new mutant CDKA;1T14V;Y15F;Thr161D (referred to as VFD). The VF mutant variant was combined with multiple CDKD alleles in which CDKD function was reduced in a step-wise fashion. This yielded a synthetically enhanced phenotype, suggesting that CDKA;1 regulates meiosis in a CDKD-dependent fashion.

The authors have taken my comments to heart and considerably improved the manuscript. The conclusions drawn by the authors are overall supported by the datasets in this revised manuscript. I have only one comment regarding the double mutant analysis (point 3) as well as minor suggestions for further improvement (points 1 & 2) that pertain to making everything absolutely explicit on the figure panels without a need for cross reference to the legend or text.

Specifically:

1. In Fig. 1 the spacing and the continuous line (a little confusing to me) in Fig. 1E do not make it fully transparent that the graphs in Fig. 1E correspond to the panels in Figure 1B-E. The panels could be connected somehow to the graphs by some discrete lines or boxes to form four clear entities, each

consisting of an image and a graph, each box clearly labelled once, and the size adjusted for a perfect alignment.

2. In general any marker used should be written somewhere (for example above) around or on each panel resulting from imaging this marker- for example in Figure 5: RFP:TUA5 and GFP:SYP132 should be clearly denoted on the figure.

3. For the description of synthetic enhancement, the authors need not put the dotted line back in if they found this confusing. However, the author's response to my concerns in the rebuttal is compelling and clear and belongs in both the results and, in an abbreviated form, in the figure legends. Would it be possible for the authors to state what an additive phenotype would be expected to be and to make it clear that what is seen is considerably stronger than an additive phenotype, thus making a clear case for a synergistic genetic interaction. I might have missed this but it did not seem explicit in the text?

Please note that I distinguish between (i) a clear conclusion that what is seen is a case of synthetic enhancement or synergy and (ii) a conservative INTERPRETATION of this finding as said before - the two gene products act IN CONCERT but not necessarily in the same pathway. On page 8 the authors have not amended this, the text still reads as follows:

"Another possibility to get insights whether two genes act in the same pathway is by asking whether specific alleles ....give rise to a synthetic ...phenotype".

The statement is flawed for the following reasons:

1. The genes do not act, the gene products do
2. "In the same pathway" could correspond to epistasis as well as to synergy
3. Synergy can be interpreted as functional redundancy, members of a protein complex, parallel pathways....

The authors could write an open sentence as follows: "To assess the nature of a possible functional interaction between the two genes, we undertook a double mutant analysis."

**Point by point response to the editors and reviewers comments**

**Editors**

**Thank you for submitting your revised manuscript entitled "CDKD-dependent activation of CKDA;1 controls microtubule dynamics and cytokinesis during meiosis". The manuscript has been seen by the original reviewers whose full comments are appended below. While the reviewers continue to be overall positive about the work in terms of its suitability for JCB, some important issues remain.**

**Both reviewers appreciate the significant revision effort and agree the manuscript is improved. While Rev #1 remains concerned about the level of novelty, Rev #2 and our editorial view is that this issue should not limit publication. Thus, we would like to invite you to address the remaining points related to the text and figures raised by the reviewers and return the manuscript to us for final consideration.**

**One point we would like you to consider when preparing the final revision is that the journal targets a broad cell biological audience. Thus, we would encourage use of nomenclature and labeling that is as accessible as possible, together with explication of system-specific features for a non-familiar reader. We encourage use of widely understood names (like "Cdk1") together with organism-specific gene names in superscript (or some analogous system) to help ensure that nomenclatural & system differences do not limit interest in and impact of the work.**

We are very happy to make our paper more accessible to the broad cell biological audience of JCB. However, as discussed with Anne Marie O'Donnell, we find it difficult to add in superscript the mammalian/yeast homologs since we sometimes have more paralogs in plants, e.g. for Cdk7/CAK there are three CDKD genes while in other occasions, we have more paralogs in animals, e.g. CDKA;1 representing Cdk1 and Cdk2. Thus, we would assign one regulator in one species to a few in another species and we fear that this is confusing as we for instance see that CDKD3

is more important in meiosis than CDKD2 and CDKD1 but all three of them would have the denominator Cdk7 in superscript. We have now added a statement about each gene we are dealing with (really largely only Cdk1 and Cdk7 homologs) explaining the relationship. In any case, if the editors have an easier and more direct way to deal with this, we are very happy to adopt this.

**Please also attend to the following formatting changes for resubmission:**

**- Provide the main and supplementary texts as separate, editable .doc or .docx files**

Separate and editable .docx files are provided.

**- Provide main and supplementary figures as separate, editable files according to the instructions for authors on JCB's website paying particular attention to the guidelines for preparing images and blots at sufficient resolution for screening and production**

Separate main and supplementary figures as .pptx are provided.

**- Provide tables as excel files**

Excel table of primers used in this study are provided.

**- Add paragraph after the Materials and Methods section briefly summarizing all "Online Supplementary Materials"**

"Online Supplementary Materials" was added.

**Reviewer 1**

**This is a revised manuscript by Sofroni et al. In response to my original review the authors added an extensive dataset demonstrating interaction between CDKA and Cyclin B, performed additional control experiments for the co-localization of CDKD and microtubules during metaphase I and metaphase II, provided an additional dataset to characterize the phragmoplast-like structures that form prior to NEB in prophase, added localization of the anti-parallel microtubule bundling factor MAP65-3 during meiosis. A significant number of figures and supplemental data were added, and corresponding textual changes**

**done. I highly appreciate the efforts that authors invested in the revised manuscript.**

We thank this reviewer for acknowledging our work and for spending once more time and energy to help improving our manuscript.

**Overall this paper contains a high-quality (and large) dataset. The volume of genetic analyses is very impressive. Clearly, it was a very laborious project. However, the JCB manuscripts are judged by novelty of the mechanistic insights into cellular processes and by their interest to the broad cell biology community. Identification of specific cyclin is highly appropriate for the storyline, but this finding is not surprising considering that Cyclins are known to function as CDK activators.**

As the reviewer may know, there is a large family of cyclins in Arabidopsis with a total of more than 30 genes. Thus, we think it is not so trivial and simple that we have identified here a cyclin partner that acts together with CDKA;1 to regulate microtubules. Please note also that we put a lot of effort into the functional characterization of CYCB3;1 including a newly developed drug assay. We also think that this assay will be helpful in general for people studying meiosis.

**I lack sufficient expertise on the protein phosphorylation during yeast or mammalian cell division to verify the claim in the rebuttal letter, but identification of a substrate would significantly advance our understanding of the signalling pathways during meiosis.**

We fully agree that the identification of a substrate would be a major step forward. However, as we tried to explain this is not a simple task for several reasons. First, it is difficult to obtain enough material from meiocytes, which are deeply buried in the plant reproductive organs. It is specially challenging to obtain enough meiocytes in cytokinesis, which is only short phase during meiosis. Second and perhaps more crucial, the mere determination of a phosphorylated protein is not sufficient evidence to claim that this phosphorylation is of biological relevance. Mutants for potential

substrates are needed, if there is redundancy, multiple mutants have to be created and then complemented with phospho-mimicry versions of the protein and/or version in which the phosphorylated amino acid(s) are substituted with amino acid that cannot be phosphorylated. The resulting phenotypes have to be carefully analyzed.

Phosphomimicry version have to be tested to what degree they can compensate for the loss of the respective kinase activity, etc. This by itself is a full-blown paper if done thoroughly.

Please note that our focus here was on the presentation of CDKDs/Cdk7 as important regulators of meiosis. To our understanding, this is also new in animals. Moreover, we reveal CDKA;1 in conjunction with CYCB3;1 as a major regulator of microtubules in meiosis. Again, we think that this is new for animals and plants. As mentioned above, we have developed a new tool to address this and other questions in meiosis. Finally, we demonstrate the activity dependency of cytokinesis on Cdk activity.

**Without an idea about which pathways are affected in the mutants, some conclusions seems too speculative. For example, the physiological relevance of altered CDK activity in the context of transition from simultaneous to sequential cell division is questionable. Are species with sequential division have reduced CDK activity? Localization of MAP65-3 shows no discernible modifications in the mutant background and as far as I can judge from the figures, MAP65-3 is unlikely involved in the above change of the cell division.**

We would like to stress that little is known about the molecular regulation of meiotic cytokinesis. Our work indeed raises the question whether crops have lower levels of CDK activity and hence develop a successive rather than a synchronous cytokinesis. We think this is an interesting hypothesis that can be tested in the future (although such a cross-species comparison is not so easily done) and hence, we hope that our work is stimulating for a large audience.

We also like to emphasize that MAP65-3 does shows an altered localization pattern (in the double *VF cdka;1 cdkd;3* mutant background). In figure 6E-I, MAP65-3 as it decorates bundled microtubule before nuclear envelope breakdown, a situation not observed in the wildtype. We did not conclude that the changes in the cell division



program are due to MAP65-3, but as this protein has several CDK phosphorylation sites it might be indeed be a bona fide substrate. Please note that this work was included as possible hint at CDK substrates as requested by the reviewer. Again, the functional proof of this is not trivial and need much more experimentation.

**Another essential issue remains association of CDK with microtubules during meiosis. I commented on the lack of appropriate negative and positive controls. In the revised manuscript mVenus driven by CDKA;1 promoter was used as a negative control and the data included in Figure 7A. The image acquisition parameters in this experiment were apparently optimized for the neighbouring tapetum cells that have strong mVenus signal in the nucleus. For this reason cytoplasmic signal in the meiotic cells appears weak. Adjusting the levels in this image produced a similar fluorescence signal in the spindle region as shown for these stages in Figure 7B. Given that, I'm not convinced mVenus signal does not accumulate around spindle microtubules. Proving specific localization of proteins on the spindle, unless there is direct association with microtubules, is technically challenging as this cytoplasm-rich region naturally accumulates almost every cytoplasmic protein. A strongly expressing control is necessary. Also measuring association of the CDKA;1 with spindle microtubules versus a strongly-expressed control would strengthen this conclusion.**

We want to emphasize that the image acquisition parameters were the same for both Figure 7A and 7B and were not adjusted to expression levels in tapetum cells. Based on the previous reviewer comments, we used the CDKA;1 promoter as a control. However, as mentioned in our previous response, the mVenus signal in meiocytes was weaker than expected. Possibly, introns of the genomic reporter of CDKA;1 enhance its expression. None-the-less, we still think that our analyses show that CDKA;1 is enhanced at the spindle, please see Figure 7B and the quantification there in. However, to respond to this reviewer, we have down-tuned our conclusion in the manuscript and indicate that a the association of CDKA;1 with the spindle is weak. However, we still believe that this interaction is real and biologically important since CYCB3;1 is very clearly associated with the spindle (please see figure 8).

Importantly, we see spindle defects when reduced CDKA;1 activity and in cycb3;1 mutants treated with Oryzalin and reference to these results now in this section

**Following from the above, proving association between mitotic spindle and CDKA;1 may not be necessary. As shown in Figure 6, the "phragmoplast-like structures" in the mutant appear already during pre-prophase before the NEB and spindle assembly. Hence, the observed defects in the microtubule dynamics and the sequential cytokinesis may not require association between the kinase and microtubules. The regulatory phosphorylation events could occur on cortical microtubules, on plasma membrane, or in the cytoplasm.**

We are not entirely sure where the reviewer is aiming at with this comment. We see meiotic spindle defects and premature microtubule structures that we have described as phragmoplast like (see comment below). Both defects could be caused in a very indirect manner from cortical microtubules. However, as mentioned above, we see that CDKA;1 and very clearly CYCB3;1 are associated with the spindle and reducing their activity causes spindle defects. Thus, the likeliest explanation is that there is a direct interaction and regulation. However, we agree that we cannot exclude an indirect effect or at least a partial involvement of an indirect effect and mention this now in the revised text.

**In this version the authors call microtubule bundles in the prophase the "phragmoplast-like structures" because they are labelled by MAP65-3. First, anaphase spindle midzone also accumulates MAP65-3. It means presence of this marker alone does not warrant defining these structures as the phragmoplasts, though I also find this rather tempting. Second, the phragmoplast is characterized by deposition of membrane to the midzone. The membrane visualization in Figure 5D shows membrane deposition only during cytokinesis. The most accurate would be to call these structures anti-parallel microtubule bundles. Furthermore, semantically "phragmoplast-like structure" sounds confusing as it implies cells skip metaphase and anaphase to proceed into cytokinesis while having intact nuclear envelope.**

We agree that these structures are best described as anti-parallel microtubule bundles as seen in phragmoplasts and we have adopted this phrase in the revised version.

#### **Reviewer 2**

**The major conclusion of this study is that a reduction in CDKA;1 activity results in a shift from synchronous to successive cytokinesis during male meiosis. This is interesting from both evolutionary and functional perspectives as synchronous cytokinesis (one cytokinesis giving rise to four gametes) occurs in the model organism Arabidopsis whereas successive cytokinesis (with a cell division after meiosis I and meiosis II) occurs in some crop species such as maize. CDKA;1 activity was slightly reduced in a previously characterized mutant CDKA;1T14V;Y15F (referred to as VF) and more substantially reduced in a new mutant CDKA;1T14V;Y15F;Thr161D (referred to as VFD). The VF mutant variant was combined with multiple CDKD alleles in which CDKD function was reduced in a step-wise fashion. This yielded a synthetically enhanced phenotype, suggesting that CDKA;1 regulates meiosis in a CDKD-dependent fashion.**

**The authors have taken my comments to heart and considerably improved the manuscript. The conclusions drawn by the authors are overall supported by the datasets in this revised manuscript. I have only one comment regarding the double mutant analysis (point 3) as well as minor suggestions for further improvement (points 1 & 2) that pertain to making everything absolutely explicit on the figure panels without a need for cross reference to the legend or text.**

We would also like to thank this reviewer for her/his constructive comments on our work and the invested time.

#### **Specifically:**

**1. In Fig. 1 the spacing and the continuous line (a little confusing to me) in Fig. 1E do not make it fully transparent that the graphs in Fig. 1E correspond to the panels in Figure 1B-E. The panels could be connected somehow to the graphs by**

**some discrete lines or boxes to form four clear entities, each consisting of an image and a graph, each box clearly labelled once, and the size adjusted for a perfect alignment.**

We rearranged figure 1 by connecting the graphs with the respective panels by discrete lines as proposed by the reviewer.

**2. In general any marker used should be written somewhere (for example above) around or on each panel resulting from imaging this marker- for example in Figure 5: RFP:TUA5 and GFP:SYP132 should be clearly denoted on the figure.**

We have double-checked all the figures to assure that they all have the marker line written in each panel.

**3. For the description of synthetic enhancement, the authors need not put the dotted line back in if they found this confusing. However, the author's response to my concerns in the rebuttal is compelling and clear and belongs in both the results and, in an abbreviated form, in the figure legends. Would it be possible for the authors to state what an additive phenotype would be expected to be and to make it clear that what is seen is considerably stronger than an additive phenotype, thus making a clear case for a synergistic genetic interaction. I might have missed this but it did not seem explicit in the text?**

We appreciate this comment of the reviewer. For us, the case was clear since all homozygous single mutants have around 5% defect, some of them even none. In contrast, the homozygous double mutant combinations have in 60 to even 100% of all cases defects. However, it is true that we never clearly stated this and we agree that such a conclusion helps the reader to understand our arguments. We have now added a paragraph explicitly stating that the observed defects go much beyond any additive effects.

**Please note that I distinguish between (i) a clear conclusion that what is seen is a case of synthetic enhancement or synergy and (ii) a conservative INTERPRETATION of this finding as said before - the two gene products act IN CONCERT but not necessarily in the same pathway. On page 8 the authors have not amended this, the text still reads as follows:**

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- 2. "In the same pathway" could correspond to epistasis as well as to synergy**
- 3. Synergy can be interpreted as functional redundancy, members of a protein complex, parallel pathways....**

**The authors could write an open sentence as follows: "To assess the nature of a possible functional interaction between the two genes, we undertook a double mutant analysis."**

We thank the reviewer for this suggestion and changed the text accordingly.