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Cuf2 boosts the transcription of APC/C activator Fzr1 to terminate the meiotic division cycle

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Transaction Report:

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

1st Editorial Decision 23 December 2012

Thank you for the submission of your research manuscript to EMBO reports. We have now received the full set of referee reports on it, which I copy below.

As you will see, the referees agree that the study is potentially interesting. Referees #2 and #3 are overall positive about the work suggesting minor changes plus further statistical analysis of the characterization of the mutant phenotypes. However, referee #1 and in particular referee #4 raises serious concerns pertaining to the claim that Sms5/Cuf2 mutants undergo a third meiosis. Referee #4 therefore feels that the main conclusion of the study is not supported by the data provided. In addition referee #1, after we invited reviewers to cross comment on each other's reports, a standard procedure for all our decisions, agreed with referee #4. She/he indicates that further examination into the structure of the spindles produced in the defective meiosis round, would be necessary to make the study fully conclusive.

Given the potential interest of the novel findings, I would like to give you the opportunity to revise

the manuscript, with the understanding that the referees' concerns, in particular those of referee #4 and #1, have to be fully addressed as this would be essential for the conclusiveness of the study. Acceptance of the manuscript would entail a second round of review. I would like to point out that it is EMBO reports policy to allow a single round of revision only, and that thus acceptance or rejection of the manuscript will depend on the outcome of the next final round of peer-review.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. If you feel that this period is insufficient to address the referees' concerns I can potentially extend this period slightly. Also, the length of the revised manuscript should not exceed roughly 30,000 characters (including spaces). Should you find the length constraints to be a problem, you may consider including some peripheral data in the form of Supplementary information. However, materials and methods essential for the understanding of the key experiments should be described in the main body of the text and may not be displayed as supplemental information only.

We have also started encouraging authors to submit the raw data for western blots (i.e. original scans) to our editorial office. These data will be published online as part of the supplementary information. This is voluntary at the moment, but if you agree that this would be useful for readers I would like to invite you to supply these files when submitting the revised version of your study.

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

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

I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

REFEREE REPORTS:

Referee #1

Report on Aoi et al; pombe sms5

This is an interesting paper, which address the question of how meiosis is terminated. The authors perform a genetic screen, to find mutants that restore sporulation (and by implication completion of meiosis) to the mes1 mutant. They propose that increased expression of the APC/C cofactor fzr1, mediated by sms5, participates in the termination of meiosis.

Overall, the data are of high quality, but in my view, a few points require attention before publication can be considered.

First, since sms5 is an allele of cuf2, why is it necessary to rename the gene in this study? If this mutant in cuf2 is specific to meiosis in its effects, they could give it allele name to reflect this. There are already a large number of genes for which there are multiple names: adding to it is not desirable, in my view.

Second: is the sms1/fzr allele isolated in the screen a loss of function? The study mostly uses the fzr1D strain. If this is a separation of function allele, it might be of interest to those studying the

structure of the APC/C accessory proteins.

Third: previous reports of monopolar spindles such as those made in cut7, cut12, or sad1, for example, show a "crow's foot" spindle. In this case, there seems only to be a single band of microtubules emanating from the SPB. Is there any idea of why this should be so?
Also, in this mutant, is there any evidence for FSM production at the SPB prior to the "meiosis III". Since they claim that the kinetics of meiosis are normal to that point, it would be interesting to know if FSM production is initiated at all.

Fourth: it would be useful to know how the binding of cuf2/sms5 to fzr1 compares to a well-establish target of cuf2.

Fifth: Figure 4: The conclusion that cdc13 persists into the putative meiosis III is not supported by the data shown. I do not dispute that fact that cdc13 persists for longer in the sms5D background, but in the images provided, it is not clearly detectable after 3.5h, which is before the appearance of cells with 5 or more nuclei. It might be helpful to provide a quantification of the western blot, relative to tubulin, for example.
It is also clear that the fzr1 protein appears at a low level earlier than usual in the sms5D. This may point to a role of cuf2 as a repressor of the fzr1 expression. This point should be explored further.

Referee #2

The manuscript by Aoi et al describes a novel meiotic phenotype for mutants in regulators of the APC in fission yeast meiosis. Both fzr1 and sms5/cuf2 mutants attempt to undergo a "third" meiotic division. The authors show that sms5/cuf2 directly regulates the expression of fzr1 and provide evidence that differential levels of APC regulate the transition of MI to MII and then allow for termination of the meiotic divisions. These results are of interest to a broad range of readers investigating meiosis and cell cycle control. I have a few recommendations to improve the manuscript:
1) statistics should be applied to the analysis of the terminal phenotypes of the single and double mutants. This is particularly important when concluding that the phenotype of the double mutant is no different than the fzr1 single mutant.
2) It would be nice to include the model as part of the main manuscript instead of in the supplemental results. If there is a space issue, it could be incorporated into figure 1A.
3) The writing could be improved.

Referee #3

It is a fundamental feature that meiosis is composed of two consecutive nuclear divisions, meiosis-I and meiosis-II. This is a nicely performed work with fission yeast to show that how the meiotic division is restricted to only two rounds of division. The authors discovered two genes, sms5 and sms1/fzr1/mfr1, which are involved in this restriction of meiotic division. They found that the additional division (they termed meiosis III-like division) was partially induced in either sms5, fzr1 or double mutant cells. Mfr1 has already been identified as the coactivator of APC/C. mfr1 mutant cells failed to degrade B-type cyclin, Cdc13, after meiosis-II. Sms5 is a meiosis-specific transcription factor that is responsible for activation of mfr1 transcription. They also demonstrated that the ectopic spindle assembly and partial chromatin separation after completion of meiosis II by fluorescence microscopy.
Most experiments were properly designed and conducted. The results are clear and faithful. The manuscript is concise and simple to understand. Although the molecular function of Fzr1 in controlling the APC/C activity has already been worked out by other groups (e. g., Kimata et al paper in MBoC, 2011), the essential part of this study made some contribution to our understanding of general feature of meiosis.
I recommend the publication of this manuscript in EMBO Report after the following minor issues will be properly revised.

Minor points:
1. In some fungi, post-meiotic mitosis is conducted, resulting in eight-spored asci. This famous phenomenon seems to be related to the aberrant nuclear division found here. I wonder if this fact may be discussed in brief.
2. page 7, line 6, "APC/C inhibitor" should be "APC/C activator".
3. In Figure 3D, iodine staining is used to show sporulation ability of mutants. I am afraid that only yeast researchers are familiar with this method. Some explanation is needed for general readers.
4. Percent of cell type in Fig. 1C, Fig.2A etc. was calculated on diploid zygotic cell basis. To avoid confusion, it should be noted.

Referee #4

Aoi et al.: S. pombe Sms5 boosts transcription of APC/C activator Fzr1 to terminate the meiotic division cycle

Meiosis consists of one round of DNA replication followed by two consecutive rounds of chromosome segregation, called meiosis I and -II. How meiotic cells ensure that premeiotic S phase is followed by two, and only two, nuclear divisions is a fundamental but still unresolved question. Mutations leading to additional rounds of chromosome segregation have been identified in mouse and plants but these studies have not led to a general concept for the underlying control mechanisms. Aoi et al. have attempted to investigate this problem in fission yeast by screening for suppressors of a deletion of the mes1 gene. At the meiosis I-to-meiosis II transition, Mes1 inhibits APC/C-Cdc20 and the meiosis-specific APC/C-Fzr1 (Fzr1 is also known as Mfr1) to allow the re-accumulation of cyclin B (Cdc13 in fission yeast) and thereby entry into meiosis II. mes1 mutants undergo a single division and arrest in a prophase II-like state because APC/C activity prevents the accumulation of cyclin B required for entry into meiosis II. Aoi et al. reasoned that a mutation, which restores the meiosis II division in a mes1 mutant, might cause additional divisions in otherwise wild-type cells. They find that deletion of fzr1 or cuf2/sms5 restores the meiosis II division in mes1 mutant cells. Restoration of meiosis II in the mes1 mutant by deletion of fzr1 has been discovered and analyzed in detail previously (Kimata et al., 2011, Mol. Biol. Cell 22). Cuf2/Sms5 has been described previously as a meiosis-specific transcription factor (Ioannoni et al., 2012, PlosOne 7) and is shown here to enhance the expression of fzr1. Aoi et al. might want to acknowledge the previous description of cuf2 by referring to this gene as cuf2 rather than sms5.
Previous work has shown that Fzr1/Mfr1 is required for rapid degradation of cyclin B at the end of meiosis II and for spore wall formation (Blanco et al., 2001, J. Cell Sci. 114; Asakawa et al., 2001, Mol. Genet. Genomics 265). fzr1 deletion mutants complete the meiosis II division but cyclin B levels remain elevated. Furthermore, formation of forspore membranes, the precursor structures of the spore wall, is defective. Importantly, a fraction of the mutant cells contain too few forspore membranes (resulting in some "naked" nuclei) or too many (resulting in forspore membranes without nuclei). Even forespore membranes that encapsulate a nucleus fail to form a spore wall. A similar phenotype has been shown to result from the deletion of cuf2 (Ioannoni et al., 2012).
While previous work concluded that fzr1/mfr1 deletion mutants arrest with elevated cyclin B after meiosis II, Aoi et al. claim that a fraction of the mutant cells undergo an additional round of chromosome segregation (called meiosis III). This conclusion is based on the appearance of supernumerary (i.e. >4) DNA masses and the continued ability of spindle pole bodies (SPBs) to nucleate microtubules. However, in the view of this reviewer, the experiments of Aoi et al. present no evidence for an additional round of chromosome segregation or nuclear division. Rather, the appearance of >4 DNA masses can be easily explained by the defect in forspore membrane formation and the persistence of elevated Cdk1-cyclin B activity.
Several decades of cell cycle research have established the notion that a new round of chromosome segregation requires first a drop in Cdk1 activity (due to cyclin proteolysis triggered by the APC/C) followed by a rise in Cdk1 activity (due to stabilization and synthesis of cyclin B). This oscillation of Cdk1 activity is expected to induce cellular events such as reduplication of SPBs and disassembly/re-assembly of spindles. Furthermore, a cycle of APC/C activation/inactivation should lead to the degradation and subsequent re-accumulation of crucial APC/C substrates such as securin (Cut2 in fission yeast). There is no evidence in this manuscript that any of these events occur after meiosis II in the fzr1 mutant cells. In fact, the elevated levels of cyclin B in the fzr1 mutant should be sufficient to prevent any further division. Indeed, Blanco et al. (2001) have recapitulated the fzr1

deletion phenotype by expressing a non-degradable version of cyclin B (Cdc13) in otherwise wild-type cells.
In summary, this reviewer feels that the main conclusion of Aoi et al. is not supported by the experiments presented. This conclusion is also inconsistent with generally accepted concepts of cell cycle control by Cdk1 and the APC/C. As shown previously by Akasawa et al. (2001), Blanco et al. (2001), and Ioannoni et al. (2012), fzr1/mfr1 mutants and cuf2 mutants fail to encapsulate some of their nuclei into forspore membranes after meiosis II. Due to persistent Cdk1 activity, SPBs continue to nucleate microtubules, which then pull chromosomes out of these naked nuclei, leading to the appearance of supernumerary DNA masses. This does not constitute an additional attempt at nuclear division, which might warrant the designation "meiosis III". Therefore, this reviewer feels that this manuscript is not suitable for publication. The authors should build on their finding that Cuf2 is required for normal induction of Fzr1 expression, which, in turn, is important for normal spore formation.

1st Revision - authors' response 25 February 2013

Aoi et al.
Point-by-point response to comments by the referees:

Referee #1

This is an interesting paper, which address the question of how meiosis is terminated. The authors perform a genetic screen, to find mutants that restore sporulation (and by implication completion of meiosis) to the mes1 mutant. They propose that increased expression of the APC/C cofactor fzr1, mediated by sms5, participates in the termination of meiosis.

Overall, the data are of high quality, but in my view, a few points require attention before publication can be considered.

First, since sms5 is an allele of cuf2, why is it necessary to rename the gene in this study? If this mutant in cuf2 is specific to meiosis in its effects, they could give it allele name to reflect this. There are already a large number of genes for which there are multiple names: adding to it is not desirable, in my view.
Our reply:
According to the comment, we have changed sms5 to cuf2 throughout the manuscript except for the mutant screening section, where the isolation of mes1 suppressors was described.

Second: is the sms1/fzr allele isolated in the screen a loss of function? The study mostly uses the fzr1D strain. If this is a separation of function allele, it might be of interest to those studying the structure of the APC/C accessory proteins.
Our reply:
The sms1/fzr1 mutant we isolated contains a single mutation in the promoter region of the *fzr1+* gene. We have seen that the transcription of *fzr1+* is reduced in this mutant. Thus, the sms1/fzr1 allele is likely to be hypomorphic or loss-of-function, rather than separation-of-function. This is indicated in page 4.

Third: previous reports of monopolar spindles such as those made in cut7, cut12, or sad1, for example, show a "crow's foot" spindle. In this case, there seems only to be a single band of microtubules emanating from the SPB. Is there any idea of why this should be so?

Also, in this mutant, is there any evidence for FSM production at the SPB prior to the "meiosis III". Since they claim that the kinetics of meiosis are normal to that point, it would be interesting to know if FSM production is initiated at all.

Our reply:

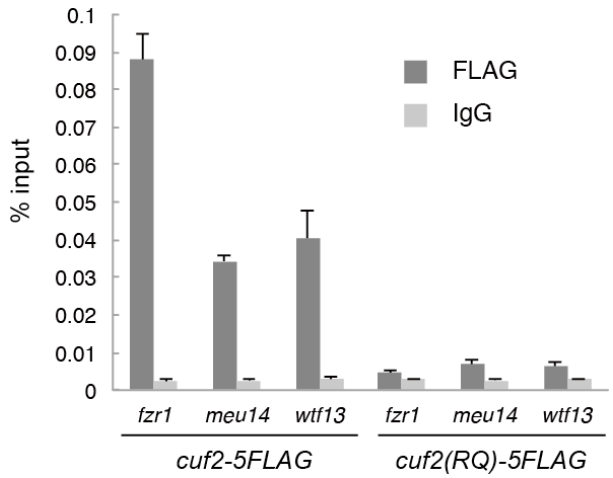
Our original explanation might have been confusing. In fact, some fzr1Δ cells exhibited V-shape (crow's foot) spindles as in the cut7 mutant, and some showed singly-bundled monopolar spindles, as was shown in the original Figure 2C,D (Figures 2C and S1A in the revised manuscript). An example of the V-shaped spindle is now shown in Figure S1C. The percentage of nuclei with a V-shaped spindle was 23%, and the statistic data including this are summarized in Table S3.

Concerning the forespore membrane (FSM), we have monitored the formation of FSM in MII onwards in cuf2Δ and fzr1Δ mutant cells, using GFP-Psy1 as a marker. As is shown in Figure S2, we have seen that FSM begins to assemble at the early stage of MII in these mutants. Thus, the meiotic development appears to be normal until MII. It is long after MII that the nuclei generate MIII-type spindles, and the ectopic spindle was assembled within a sphere of the forespore membrane. This is now described in page 5.

Fourth: it would be useful to know how the binding of cuf2/sms5 to fzr1 compares to a well-establish target of cuf2.

Our reply:

We are afraid that there may be no 'well-established' targets of Cuf2 yet, since the original paper describing Cuf2 was published only recently and this transcription factor does not appear to have much to do with copper signaling (Ioannoni et al., 2012, PLoS ONE). To address the referee's comment, we arbitrarily chose two genes, namely meu14+ and wtf13+ listed in the paper, as possible targets of Cuf2. Ioannoni et al. have shown that the expression of meu14+ and wtf13+ was higher in cuf2Δ than in WT (i.e., Cuf2 functions as a repressor). We investigated Cuf2 binding to the meu14+ and wtf13+ genes. The results were similar to the cases with cdc13+ and mes1+, i.e., Cuf2 bound to them to some degree but less strongly than to fzr1+. We show these data below as a reference for the referees, but we have decided not to include them in the manuscript, because they do not lead to solid conclusions by themselves.



Reference Figure. ChIP assays for *meu14* and *wtf13* promoters.

Cuf2–5FLAG and Cuf2(RQ)–5FLAG proteins were immunoprecipitated from cells at meiosis I/meiosis II transition with the anti-FLAG antibody. Quantitative PCR was performed to measure the amount of DNA fragments (promoter regions of respective genes) co-purified with each protein. Error bars represent s.d. (of 3 reactions).

Fifth: Figure 4: The conclusion that cdc13 persists into the putative meiosis III is not supported by the data shown. I do not dispute that fact that cdc13 persists for longer in the sms5D background, but in the images provided, it is not clearly detectable after 3.5h, which is before the appearance of cells with 5 or more nuclei. It might be helpful to provide a quantification of the western blot, relative to tubulin, for example.
Our reply:
We are sorry that the quality of the images in the original manuscript was rather poor. We repeated time-course experiments and Western blotting assays, and are now able to provide improved images, which may be convincing even without a quantification, as shown in Figure 4A,B. From these panels it will be evident that Cdc13 in cuf2Δ cells persists beyond MII, until 4.75 h, when cells with 5 or more nuclei emerge.

It is also clear that the fzr1 protein appears at a low level earlier than usual in the sms5D. This may point to a role of cuf2 as a repressor of the fzr1 expression. This point should be explored further.
Our reply:
We thank the referee for precise observation. In repeated experiments, however, we have seen that the amount of Fzr1 is generally lower in the cuf2Δ mutant than in WT, as demonstrated in Figure 4A, B. Thus, Cuf2 does not seem to function as a repressor for the expression of fzr1.

Referee #2

The manuscript by Aoi et al describes a novel meiotic phenotype for mutants in regulators of the APC in fission yeast meiosis. Both fzr1 and sms5/cuf2 mutants attempt to undergo a "third" meiotic division. The authors show that sms5/cuf2 directly regulates the expression of fzr1 and provide evidence that differential levels of APC regulate the transition of MI to MII and then allow for termination of the meiotic divisions. These results are of interest to a broad range of readers investigating meiosis and cell cycle control. I have a few recommendations to improve the manuscript:
1) statistics should be applied to the analysis of the terminal phenotypes of the single and double mutants. This is particularly important when concluding that the phenotype of the double mutant is no different than the fzr1 single mutant.
Our reply:
Following the referee's advice, we now include statistics (P value of Student's t test) regarding the percentages of cells with 5 or more nuclei in the legend to Figure 2A. The results confirm that the frequencies of cells with ≥5 nuclei in fzr1Δ and in cuf2Δ fzr1Δ are not significantly different.

2) It would be nice to include the model as part of the main manuscript instead of in the supplemental results. If there is a space issue, it could be incorporated into figure 1A.

Our reply:
According to the comment, we have moved the model figure (formerly Figure S2) to Figure 4F.

3) The writing could be improved.
Our reply:
The revised manuscript has been reedited thoroughly by a native English speaker.

Referee #3

It is a fundamental feature that meiosis is composed of two consecutive nuclear divisions, meiosis-I and meiosis-II. This is a nicely performed work with fission yeast to show that how the meiotic division is restricted to only two rounds of division. The authors discovered two genes, sms5 and sms1/fzr1/mfr1, which are involved in this restriction of meiotic division. They found that the additional division (they termed meiosis III-like division) was partially induced in either sms5, fzr1 or double mutant cells. Mfr1 has already been identified as the coactivator of APC/C. mfr1 mutant cells failed to degrade B-type cyclin, Cdc13, after meiosis-II. Sms5 is a meiosis-specific transcription factor that is responsible for activation of mfr1 transcription. They also demonstrated that the ectopic spindle assembly and partial chromatin separation after completion of meiosis II by fluorescence microscopy.
Most experiments were properly designed and conducted. The results are clear and faithful. The manuscript is concise and simple to understand. Although the molecular function of Fzr1 in controlling the APC/C activity has already been worked out by other groups (e. g., Kimata et al paper in MBoC, 2011), the essential part of this study made some contribution to our understanding of general feature of meiosis.
I recommend the publication of this manuscript in EMBO Report after the following minor issues will be properly revised.

Minor points:
1. In some fungi, post-meiotic mitosis is conducted, resulting in eight-spored asci. This famous phenomenon seems to be related to the aberrant nuclear division found here. I wonder if this fact may be discussed in brief.
Our reply:
It is an interesting question how post-meiotic mitosis is regulated in fungi that produce eight-spored asci. It could be possible that they restrict the APC/C activity after MII so that another round of nuclear division may take place, as in the S. pombe cuf2 and fzr1 mutants analyzed in this study. We have briefly mentioned this possibility in the discussion section of the revised manuscript (page 8).

2. page 7, line 6, "APC/C inhibitor" should be "APC/C activator".
Our reply:
We apologize for the mistake. It is now corrected.

3. In Figure 3D, iodine staining is used to show sporulation ability of mutants. I am afraid that only yeast researchers are familiar with this method. Some explanation is needed for general readers.

Our reply:
We thank the referee for the advice. We now explain iodine staining in the legend to Figure 3D.

4. Percent of cell type in Fig. 1C, Fig.2A etc. was calculated on diploid zygotic cell basis. To avoid confusion, it should be noted.
Our reply:
We now clarified it in the legends (Figures 1C, 2A and 3D).

Referee #4

While previous work concluded that fzr1/mfr1 deletion mutants arrest with elevated cyclin B after meiosis II, Aoi et al. claim that a fraction of the mutant cells undergo an additional round of chromosome segregation (called meiosis III). This conclusion is based on the appearance of supernumerary (i.e. >4) DNA masses and the continued ability of spindle pole bodies (SPBs) to nucleate microtubules. However, in the view of this reviewer, the experiments of Aoi et al. present no evidence for an additional round of chromosome segregation or nuclear division. Rather, the appearance of >4 DNA masses can be easily explained by the defect in forspore membrane formation and the persistence of elevated Cdk1-cyclin B activity.
Several decades of cell cycle research have established the notion that a new round of chromosome segregation requires first a drop in Cdk1 activity (due to cyclin proteolysis triggered by the APC/C) followed by a rise in Cdk1 activity (due to stabilization and synthesis of cyclin B). This oscillation of Cdk1 activity is expected to induce cellular events such as reduplication of SPBs and disassembly/re-assembly of spindles. Furthermore, a cycle of APC/C activation/inactivation should lead to the degradation and subsequent re-accumulation of crucial APC/C substrates such as securin (Cut2 in fission yeast). There is no evidence in this manuscript that any of these events occur after meiosis II in the fzr1 mutant cells. In fact, the elevated levels of cyclin B in the fzr1 mutant should be sufficient to prevent any further division. Indeed, Blanco et al. (2001) have recapitulated the fzr1 deletion phenotype by expressing a non-degradable version of cyclin B (Cdc13) in otherwise wild-type cells.
Our reply:
This referee expressed concern that, in his/her view, our original manuscript presented no evidence for an additional round of chromosome segregation or nuclear division. Given this criticism, we have tried to include more convincing data that will support the occurrence of meiosis III-like division. These data are summarized in new panels (Figures 2E,F and 4D,E) and Table S3. We now show detailed time-lapse images for the MIII-like division in fzr1Δ. One can see that the elongated spindle in MII (Figure 2F, 0~10min, fzr1Δ) underwent disassembly at 30 min. At this stage remnant microtubule fragments could be seen. At 40 min the remnants disappeared and, instead, blob microtubules with bright GFP-Atb2 signal appeared around the SPB. The blobs grew into spindles within another 40 min. The zygote shown in this panel generated one bipolar spindle, two monopolar spindles, and one monopolar spindle with SPB duplication (80 min), demonstrating the progression of a ‘microtubule cycle’ of disassembly and reassembly after MII. Statistical data concerning generated spindles are summarized in Table S3.

We also observed the behavior of securin (Cut2-GFP) in fzr1Δ, which stays on metaphase spindles and is a target of APC/C, like Cdc13. As shown in Figure 4E, Cut2-GFP diminished in late MII (Figure 4E, 15~35 min), but it re-accumulated in another 35 minutes (70min). Therefore Cut2, similarly to Cdc13 demonstrated in Figure 4C, exhibits a cycle of on-off-on from MII onwards. Relevant descriptions are given in pages 5 and 7.

In summary, this reviewer feels that the main conclusion of Aoi et al. is not supported by the experiments presented. This conclusion is also inconsistent with generally accepted concepts of cell cycle control by Cdk1 and the APC/C. As shown previously by Akasawa et al. (2001), Blanco et al. (2001), and Ioannoni et al. (2012), fzr1/mfr1 mutants and cuf2 mutants fail to encapsulate some of their nuclei into forspore membranes after meiosis II. Due to persistent Cdk1 activity, SPBs continue to nucleate microtubules, which then pull chromosomes out of these naked nuclei, leading to the appearance of supernumerary DNA masses. This does not constitute an additional attempt at nuclear division, which might warrant the designation "meiosis III". Therefore, this reviewer feels that this manuscript is not suitable for publication.

Our reply:

The behavior of microtubules, cyclin, and securin suggested that fzr1Δ cells were unlikely to simply continue to nucleate microtubules from SPBs after MII. To examine possible chromosome segregation, we chased the behavior of kinetochores using Mis6-2mRFP as a marker. As shown in Figure 2F, kinetochores segregated in MII at 30 min, and then they were aligned on the ectopic spindle again in fzr1Δ (80 min). This demonstrates that chromosomes also enter another round of M phase in fzr1Δ. The ectopic spindles appeared to be assembled generally within FSM (Figure S2), suggesting that chromosome segregation led by them is performed within a sphere of FSM, rather than in “naked” nuclei. Relevant descriptions are given in page 5.

Altogether, we believe that we have now presented sufficient evidence in the revised manuscript for the following conclusions, which will support our proposal of the MIII-like division.

(1) An additional round of the microtubule cycle takes place after MII in fzr1Δ.
(2) An additional round of the cyclin and securin cycle takes place after MII in fzr1Δ.
(3) Microtubule-kinetochore attachment resumes after MII in fzr1Δ, within the sphere of FSM.

The authors should build on their finding that Cuf2 is required for normal induction of Fzr1 expression, which, in turn, is important for normal spore formation.

Our reply:

We agree that Cuf2 contributes to robust spore formation through regulation of FSM assembly. However, the cuf2Δ and fzr1Δ strains revealed phenotypes that could not be explained solely by the defect in forspore membrane formation, as described above. We hope sincerely that the referee will accept our argument that such phenotypes are related to the induction of MIII-like division, caused by the residual Cdc13 activity in these mutants.

2nd Editorial Decision 06 March 2013

Thank you for your patience while your study was assessed for a second time by referees 1 and 4. As you will see, although referee 1 was initially supportive, referee 4 maintains his/her major concern, that the main conclusion regarding a third meiosis of Sms5/Cuf2 mutants has not been conclusively demonstrated. Upon further discussion, referee 1 also considered that this has not been demonstrated.

As we mentioned in our previous decision letter, addressing this point to the referees' satisfaction was a precondition for acceptance. Seeing as though there are still crucial concerns regarding the central premise of your study, we can unfortunately not offer to publish your manuscript.

I am very sorry to disappoint you on this occasion, and hope that the referee comments are helpful in your continued work in this area.

REFEREE REPORTS:

Referee #1:

Having read the revised paper, I think the authors have addressed my questions in a satisfactory manner.

Referee #4:

Revision of Aoi et al.: S. pombe Sms5 boosts transcription of APC/C activator Fzr1 to terminate the meiotic division cycle

Aio et al. claim that the deletion of the fzr1/mfr1 gene, encoding a meiosis-specific APC/C activator, causes fission yeast cells to undergo an additional meiotic division, called meiosis III. A similar, albeit weaker, phenotype is reported for the elimination of Cuf2, a transcription factor required for normal induction of the fzr1/mfr1 gene. In contrast, previous work has shown that fzr1/mfr1 mutants are severely delayed in the degradation of cyclin B (Cdc13) and, thus, fail to exit from meiosis II (Blanco et al., 2001, J. Cell Sci. 114; Asakawa et al., 2001, Mol. Genet. Genomics 265). As a consequence, the mutant cells form aberrant numbers of prospore membranes, which fail to properly encapsulate the segregated chromosomes. This phenotype could be reproduced by the expression of a version of cyclin B/Cdc13 that is resistant to APC/C-dependent degradation. The defect in prospore membrane behaviour has also been reported previously in the cuf2 mutant (Ioannoni et al., 2012).
In my view, elevated Cdk1-cyclin B activity leading to continuous nucleation of dynamic microtubules by spindle pole bodies (SPBs) together with the aberrant formation of prospore membranes is sufficient to explain the phenotypes reported by previous work and, here, by Aio et al. An additional "meiosis III" division would predict the coordinated occurrence of several events including degradation and re-accumulation of cyclin B/Cdc13 and other APC/C substrates, SPBs re-duplication (rather than occasional fragmentation), and disassembly and re-assembly of dynamic microtubules. These events are best analysed by time-lapse microscopy with high temporal resolution. Cell cycle events should be carefully quantified and the movies should be deposited in supplemental material. This type of experiment is standard in the analysis of the cell cycle progression and meiosis in fission yeast. Aio et al. do not present an analysis that is adequate for their extraordinary claim. Selected images taken at long time intervals are not sufficient to support the notion of a meiosis III division and, importantly, to refute the conclusions of previous publications.
Also, I would like to encourage the authors to discuss in more detail the findings reported in previous publications including the implications of the defect in prospore membrane formation

(rather than merely hinting at abnormal nuclear division). Finally, I note that a gene required for exit from meiosis is not necessarily required for suppression of additional divisions in meiosis. The authors should avoid raising such an expectation in readers not familiar with the intricacies of cell cycle regulation or control of meiosis. In summary, I feel that this manuscript is not suitable for publication in EMBO Reports.

Correspondence - Appeal 06 March 2013

I was really surprised to read the comments of Referee #4 and your editorial decision heavily depending on them. We have addressed all the concerns of Referee #4 raised in the previous report, and now he/she claims that our work is not suitable for publication because we do not provide a movie, which he/she never mentioned in the initial comments. We now know that three out of the four referees fully support our study (it is unfair to re-consult Referee #1 conveying Referee #4's comments, after he/she once agreed to accept the manuscript). From my experience to study fission yeast meiosis for more than thirty years, I would say that the view of Referee #4 is rather extraordinary, representing a sort of belief rather than logical considerations based on the evidence, which leads me even to suspect that he/she might have a conflict of interest.

Given that three referees judged our work affirmatively, I would like to ask you to send our manuscript to a few new referees and get their comments. It appears too unfair to us that you make a negative decision depending on a claim of a single referee, who might be eccentric. If we had known his/her name, we would have used our right to avoid him/her as a referee from the beginning.

Thank you very much for your attention and consideration.

3rd Editorial Decision 14 March 2013

Thank you for your patience while I have sought additional advice on your study. Our advisor has carefully read the study and the referees' comments and I am happy to say that s/he is supportive of publication after minor revision. We have therefore decided to overrule referee #4's remaining concerns and proceed with publication of your study.

In order to be able to accept your work, however, a few minor issues have to be addressed, as follows.

- Please supply the relevant movie/movies as supplementary information

- Our advisor agreed with referee #4 that more judicious citation of Blanco et al. and Asakawa et al. is called for, although s/he notes that these previous papers did not properly document a failure to exit meiosis II, nor show that nuclei could progress to a third prophase. Thus, please discuss these previous studies in more detail.

According to our count, your manuscript is only slightly over 20,500 characters. As you have only four figures, your could extend the text in up to 10,000 characters, which should give you plenty of space to incorporate this discussion.

- Nevertheless, our advisor considered that the phenotype observed is not a uniform meiosis III. The

cells seem to be entering something akin to prophase of meiosis III, as Cut2 securin comes back but never goes away, and the chromosome segregation observed could be due to monopolar attachment and movement to the pole in the absence of tension. Thus, although the wording throughout the text is rather careful, the mentioning of a "third division" in the abstract should be toned down. As you will see below, I have provided a suggestion for your approval.

- From an editorial standpoint, the material & methods section needs to be expanded in the main text to include the description of meiosis induction and microscopy, as methods essential to the understanding of the experiments must be described in the main body of the manuscript. Also, the number of cells analyzed seems to be missing from the legends of figures 3A and 4A,B.

Finally, As a standard procedure, we edit the title and abstract of manuscripts to make them more accessible to a general readership. Please find the edited versions at the end of this email and let me know if you do NOT agree with any of the changes. I have deleted S. pombe from the title in keeping with journal style, as people reading the abstract will immediately understand the study was performed in fission yeast. Likewise, as fission yeast is mentioned, S. pombe seemed redundant in the abstract. The name is in the third sentence of the article text, so I do not think it will be misleading and the abstract text flows better.

I look forward to seeing a final version of your manuscript as soon as possible.

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Edited title and abstract

Cuf2 boosts the transcription of APC/C activator Fzr1 to terminate the meiotic division cycle

The number of nuclear divisions in meiosis is strictly limited to two. Although the precise mechanism remains unknown, this seems to be achieved by adjusting the anaphase-promoting complex/cyclosome (APC/C) activity to degrade cyclin. Here, we describe a fission yeast cuf2 mutant that enters into a third nuclear division cycle, represented by ectopic spindle assembly and abnormal chromosome segregation. Cuf2 is a meiotic transcription factor, and its critical target is fzr1+/mfr1+, which encodes a meiotic APC/C activator. fzr1Δ also enters a third nuclear division. Thus, Cuf2 ensures termination of the M-phase cycle by boosting Fzr1 expression to generate functional gametes.
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3rd Revision - authors' response 01 April 2013

We treated each comment of the Editor as in the following.

- Please supply the relevant movie/movies as supplementary information.
We now supply six movies as supplementary information. Movies 1-4 correspond to Figures 2B, 2C, 2E and 2F, respectively. Movie 5 prepared newly is of high resolution and we hope that it will present convincing images of chromosome segregation in the third division in *fzr1*Δ. Movie 6 is a control to Movie 5.

- Our advisor agreed with referee #4 that more judicious citation of Blanco et al. and Asakawa et al. is called for, although s/he notes that these previous papers did not properly document a failure to exit meiosis II, nor show that nuclei could progress to a third prophase. Thus, please discuss these previous studies in more detail.
We have referred to these previous studies and summarized their results in page 4, as introductory remarks. We also mentioned their conclusions in connection with ours in page

8. In addition, we have cited a publication that described the involvement of *Saccharomyces* Ama1, a homolog of Fzr1, in spore wall formation, in page8.

- Nevertheless, our advisor considered that the phenotype observed is not a uniform meiosis III. The cells seem to be entering something akin to prophase of meiosis III, as Cut2 securin comes back but never goes away, and the chromosome segregation observed could be due to monopolar attachment and movement to the pole in the absence of tension. Thus, although the wording throughout the text is rather careful, the mentioning of a "third division" in the abstract should be toned down. As you will see below, I have provided a suggestion for your approval.

We are vey happy with the abstract you kindly prepared for us. In reality, however, we presume that the third division also “cycles”, because microtubules disassemble and Cut2 securin goes away after a while. We have added 105 min panels showing this to Figure 4E, but refrained from discussing about the cycling in the text on an insufficient basis. We appreciate the modest but solid tone of the abstract.

- From an editorial standpoint, the material & methods section needs to be expanded in the main text to include the description of meiosis induction and microscopy, as methods essential to the understanding of the experiments must be described in the main body of the manuscript. Also, the number of cells analyzed seems to be missing from the legends of figures 3A and 4A,B.

We have moved the description of meiosis induction and microscopy from the supplementary information to the main text. We have added the number of cells analyzed (n > 200) to the legends of Figures 3A and 4A,B.

Finally, As a standard procedure, we edit the title and abstract of manuscripts to make them more accessible to a general readership. Please find the edited versions at the end of this email and let me know if you do NOT agree with any of the changes. I have deleted S. pombe from the title in keeping with journal style, as people reading the abstract will immediately understand the study was performed in fission yeast. Likewise, as fission yeast is mentioned, S. pombe seemed redundant in the abstract. The name is in the third sentence of the article text, so I do not think it will be misleading and the abstract text flows better.

We fully agree with the title and abstract edited by you. The revised manuscript holds them.

Many thanks for your attention and trouble.

4th Editorial Decision 02 April 2013

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

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Finally, we provide a short summary of published papers on our website to emphasize the major

findings in the paper and their implications/applications for the non-specialist reader. To help us prepare this short, non-specialist text, we would be grateful if you could provide a simple 1-2 sentence summary of your article in reply to this email.

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

