Regulated Inactivation of the Spindle Assembly Checkpoint without Mitotic Spindles

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1st Editorial Decision 26 October 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been evaluated by three referees, whose reports are copied below. Although these reviewers point to the somewhat descriptive nature of the study, they nevertheless consider the main novel observation of apparently autonomous spindle checkpoint inactivation during prolonged mitotic arrest in Aspergillus potentially important and thus in principle suited for publication in The EMBO Journal. However, all of them also feel that the study would constitute a much more compelling publication if at least some additional insight into the underlying mechanism could be provided - e.g. by identifying proteins whose required synthesis may underlie the cycloheximide effects, or by additionally testing the requirements for protein degradation e.g. by APC/C and additional APC/C substrates. In this respect, in a follow-up communication referee 1 also offered the additional suggestion to look at potential involvement of NIMA, as its APC/C-independent destruction is also required for mitotic exit and as the normal microtubule binding of NIMA would be perturbed in your experimental setup (it might therefore be worth to also look if microtubule-stabilizing drugs would have similar effects or not).

I of course realize that a full elucidation of the SAC inactivation mechanism may be beyond the scope of the current study, but should you be able to at least provide some good further insights to advance the understanding of this key point of interest, then we should be able to consider a revised version of the manuscript further for publication. In this respect, I would ask you to carefully take into account also the other general and specific points of the three referees, which I am not going to repeat here as they are nicely and constructively detailed in the reports below. As you will see, a number of these points really pertain to aspects of presentation (more accessible figures and/or more definitive data to allow more decisive conclusions) and discussion/interpretation (see e.g. referee 3’s comments on the cell cycle oscillator concept, referee 2’s important issue with the somewhat
unfortunate term of 'spindle-independent mitosis', and the request of all referees to expand a little on discussing possible molecular mechanisms as well as ramifications of the findings).

I should remind you that it is our policy to allow one single round of major revision only, and that it is therefore important to diligently answer to all the points raised at this stage. When preparing your letter of response to the referees’ comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community (For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html)

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

Yours sincerely,
Editor
The EMBO Journal

REFEREE REPORTS:
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Referee #1 (Remarks to the Author):

This paper describes the study of cellular adaptation to a sustained mitotic arrest in the spindle checkpoint, in Aspergillus nidulans.

The authors show that upon exposure to drugs that depolymerize the mitotic spindle, or after genetic interference with tubulin, cells arrest in mitosis, but after approximately one hour leave the mitotic state to form a single undivided daughter cell, with unsegregated chromosomes.

This process is similar to a phenomenon earlier described in mammalian cells, called mitotic slippage, which refers to inability of mitotic cells to arrest indefinitely in the spindle checkpoint. In mammalian cells, this process had been shown to be related to slow destruction of cyclin B1, which eventually leads to mitotic exit. In the absence of a spindle, this coincides with failed cytokinesis, hence mitotic slippage leads to the formation of a 4N G1 cell.

Albeit hard to decipher from the small images, a similar process is described here for Aspergillus, in Figures 1 and 2. Figure 3 shows that also nucleolar reassembly occurs when cells return to interphase. Figure 4 shows that cyclin B1 in mitosis is first lost gradually, but just before mitotic exit its degradation becomes faster.

In principle these result are to be expected for cells that eventually leave mitosis in spindle poison to return to G1. However, in mammalian cells there is evidence that the spindle checkpoint is not inactivated in the process of mitotic slippage, whereas Figures 5 and 7 suggest in Aspergillus the checkpoint can be inactivated just before mitotic exit. Whereas the staining of kinetochore-bound Mad proteins, taken as a sole measure for an active checkpoint, is hard to decipher, especially Figure 7 seems to suggest Mad is lost from kinetochores even if cells in spindle drugs are inhibited from mitotic exit by an APC/C mutation.

This encompasses the main conceptual novelty of the paper, apart from a more detailed description of the adaptation to mitotic arrest in a filamentous fungus.

Further evidence to support the idea that the checkpoint can be switched off after 30-60 min in spindle drugs, is presented in Figure 3, where nucleolar reformation occurs (reportedly inhibited by the checkpoint), and in Figure 4 which shows that after 30 minutes in the arrest, cyclin B1 starts to be degraded more rapidly.

Altogether, this is taken as evidence that just before mitotic exit the spindle checkpoint is inactivated. The authors claim this may be a unique feature of a filamentous fungus, an organism
which may experience exposure to environmental spindle toxins naturally, and so could adapt faster to such conditions.

The presented data, although hard to decipher, confirm and provide further insight in the way cells can adapt to mitotic arrest and support some of the conclusions, but do not entirely prove that the checkpoint is really switched off. This is mainly due to the fact that the hard to measure kinetochore localization of Mad proteins is taken as the main read-out.

In the absence of biochemical data, what is missing is e.g. a proper quantitation of the effect of checkpoint inactivation, cyclin B1 destruction, in the experiment as shown in Figure 4B.

Is cyclin B1 destruction always seen as strictly biphasic, and are kinetics of cyclin B1 destruction in the last phase identical to that in normal cells? Are other APC/C substrates degraded at the right time? How much cyclin B1 (%) is lost in the earlier, slippage fase?

Further, at present, given the large variation in the ways mammalian cells may respond to a sustained mitotic arrest, I feel the difference between Aspergillus and mammalian cells is exaggerated. It is yet insufficiently clear whether mammalian cells may also be able to eventually inactivate the checkpoint, later in the cell cycle.

Therefore, it remains to be seen how different the two systems are. This should be more clearly emphasized in the beginning of the manuscript. It should also be pointed out better that the eventual mitosis without spindles is not normal and is only initiated after a considerable lag phase. Therefore, the inactivation represents an adaptation to sustained mitotic arrest in the checkpoint, which, biologically, might be highly related to the earlier described process of mitotic slippage. It is therefore unknown if this represents a unique feature of fungi or is a more common response in eukaryotes.

Nevertheless, the observation that the checkpoint might be regulated independent of cyclin B1 destruction or mitotic exit, is conceptually interesting and should be further investigated.

Now, the authors only indicate that eventual checkpoint silencing occurs independent of protein synthesis (e.g. of a Cdk inhibitory protein). No attempts are taken to identify the proteins involved, although from other systems, various candidate proteins could be considered, which are not discussed here.

Specific points:

Figure 1: Can the images be magnified and the intracellular features studied be more clearly indicated, in a drawing or with arrows?

Figure 2: Can the degree of chromatin (un)separation be shown more clearly?

Figure 4. See above, Figure 4B / Supp Figure 3 need to be extended, preferentially including additional APC/C substrates, and the cyclin B data further quantified and compared to controls.

Figure 5. Can Bub3 also be monitored? Are total fluorescent Mad2 levels similar? This should be included in this Figure.

Figure 7. Can total Mad1 levels be included and Mad2 be shown here as well, as this Figure represents a major conclusion? Is the proteasome required for checkpoint silencing?

Figure 8. Could potentially involved factors be discussed, e.g. Cdc20 levels, any orthologs of p31-comet or Usp44? Could phosphorylation of SAC components be involved here, based on literature?

Figure 9: I am not convinced this represents a novelty, I would expect that upon wash-out of the drug, the next mitosis is normal.

Referee #2 (Remarks to the Author):
This manuscript by De Souza and collaborators uncovers a new pathway involved in spindle checkpoint inactivation after a mitotic delay in Aspergillus nidulans. This finding has broad implications, including clinical relevance, and the quality of the data is impressive and obtained by direct observation in living cells. The authors do a great job characterizing mitotic exit events in this system and their dependence on checkpoint inactivation. They further show that this checkpoint inactivation mechanism surprisingly requires protein synthesis and is independent of Cdk1/cyclin B inactivation or mitotic exit. Most strikingly, the authors report that this checkpoint inactivation mechanism is reversible upon re-entry into mitosis over at least 3 polyploid cycles. The clear difference with the process of mitotic slippage in vertebrates, which apparently does not involve checkpoint inactivation, makes this a very interesting paper to be followed up. I have to say that the evidence that slippage in vertebrate mitotic cells does not require checkpoint inactivation is fairly weak, and it is based only on localization data of checkpoint proteins right after mitotic slippage, suggesting that despite their presence at kinetochores they may not be active. I am therefore highly enthusiastic for publication of the present study in EMBO J. I do however have a few concerns that would like to see addressed to meet the high standards for publication in EMBO J:

1- From the experiment described in figure 5A, although it is clear that Mad2 re-localizes to NPCs, it seems that some of it always remains associated with KTs, as depicted with the Ndc80 marker. If true, this implies that, as it happens during mitotic slippage in vertebrates, Mad2 is still localized at KTs during SIM, compromising the main conclusion of this paper. How could the authors rule out this possibility? For the sake of comparison, it would also be interesting to follow Mad2 localization during a normal mitotic exit.

2- Stating that cells undergoing SIM "complete all aspects of mitosis except for those which require spindle function", such as NUCLEAR DIVISION (=mitosis) is a counter sense to say the least. I strongly disagree about using the term SIM for "Spindle independent mitosis", since it is not indeed a type of mitosis because no nuclear division occurs. What about "spindle independent mitotic exit"?

3- Although I don't see it as a requirement for publication, taking an educated guess of possible candidates whose synthesis maybe required for SAC inactivation would have been a big plus.

4- I found intriguing that mitosis duration gradually decreases over each subsequent cycle in benomyl (fig. 6B). I think this is worth discussing in the text and explore some possible explanations. Could it be related with the dilution of a "time" factor, such as Mad2? In other words, how does the Mad2 overall pool change with ploidy?

5- One possibility to explain that protein synthesis is required for SAC inactivation is that it must counterbalance protein degradation (via the APC?). What happens if bimE7(APC1) cells at restrictive temperature and arrested in benomyl are treated with cicloheximide? Can the SAC now be inactivated with normal kinetics?

Minor remarks:

1- The SAC does not arrest mitosis until CORRECT bipolar attachments are established. The SAC will be satisfied even in the presence of some INCORRECT attachments, such as merotelic ones, as far as they are also bipolar. Please delete the word CORRECT from abstract and introduction.

2- The final statement in the introduction regarding mammalian cells is overly speculative and should be removed from this section.

Referee #3 (Remarks to the Author):

This interesting paper documents clearly that Aspergillus cells blocked by the SAC will periodically escape, carry out at least some hallmarks of another cell cycle, then arrest again for a prolonged period, carrying out this cycle repeatedly and apparently with fairly regular timing.

The paper is largely descriptive beyond establishing this basic but interesting bit of biology, but the description is interesting, especially in that the authors show that SAC components themselves
exhibit hallmarks of cyclical SAC activation/inactivation as indicated by oscillating centromeric localization. It is shown that cyclin B proteolysis can be prevented without blocking at least one occurrence of SAC inactivation - it is not shown whether the repeated cycles do occur without cyclin B proteolysis, and this is interesting and important to know (see below). It is shown that protein synthesis is required for a relatively rapid SAC inactivation although mitotic exit apparently still occurs at longer times even in cycloheximide. This experiment suffers from the usual blunt-instrument problem with global inhibition of protein synthesis; while interesting, there is no clue as to what the critical synthesized protein(s) might be.

The supposition is made that these observations are somehow related to 'the' CDK-independent oscillator described by Orlando et al. This seems too simple to me - first, the Orlando work specifically describes a cyclin-independent transcriptional oscillator, so the specific hypothesis suggested could be tested (e.g. cell-cycle-regulated transcription proceeding at the same frequency as SAC inactivation in Aspergillus), rather than just brought up as a general speculation. Second, the Orlando work describes an oscillator functioning at zero mitotic cyclin (and probably not functioning at high mitotic cyclin, based on a lot of previous work), unlike the current putative SAC inactivator functioning at high mitotic cyclin. Third, there are at least two other cyclin-Cdk-independent oscillators described, one for centrosome/spindle pole duplication in fly and other embryos and in yeast, and one for release of the yeast Cdc14 phosphatase. At least the Cdc14 one clearly functions at high mitotic cyclin levels. So a more accurate statement would be that the SAC inactivation cycle could represent another oscillator, of unknown mechanistic relationship to the others previously characterized. This then leads to an important question (as clearly discussed in the Cdc14 oscillator work): how are the cyclin-Cdk cycle and the putative SAC inactivation cycle coordinated? Because if they are NOT coordinated the cell seems to run a risk of having the SAC inactivation cycle in an 'off' state just at the time when the checkpoint is needed, making the oscillator truly counterproductive. A 'good' biological design could be to have the cyclin-Cdk cycle and the SAC inactivation cycle entrained so that maximum sensitivity to the SAC would coordinate with the time when cyclin-Cdk is promoting spindle formation. Thinking about how such coordination of a putative SAC inactivation oscillator and the cyclin-Cdk oscillator might be achieved could be helpful in directing new experiments. For example, it would be a clue if the bimE mutation that allows SAC inactivation, nevertheless prevented SAC reactivation.

Overall, I think this paper describes some very useful and intriguing initial observations, which while somewhat descriptive at present, have the possibility to lead to important new insights. Thinking more about how to place the observations in a broader context should suggest additional mechanistic and physiological experiments, that would broaden the significance and impact of the work.

1st Revision - authors' response 24 January 2011

We thank you and your reviewers for their review of our manuscript and insights into our finding that the spindle assembly checkpoint (SAC) can be inactivated without spindle formation. All three reviewers thought our manuscript was of significant interest and, based on their suggestions, clearly found our results thought provoking. We have carefully considered their suggestions and performed additional experiments which further support our conclusions and have significantly improved the manuscript. In particular, new experiments characterizing the behavior of the SAC kinase Bub1 during SAC arrest further substantiate our findings and conclusions. In addition, we have changed the term "spindle independent mitosis" (SIM) to "spindle independent mitotic exit" (SIME), as suggested by reviewer 2. As suggested by all three reviewers, we have incorporated a more detailed discussion of the mechanisms which might contribute to SAC inactivation without spindle formation.

The detailed changes to our manuscript and new experimental data are outlined in our responses to each reviewer.

Referee #1 (Remarks to the Author):
This paper describes the study of cellular adaptation to a sustained mitotic arrest in the spindle checkpoint in Aspergillus nidulans.

The authors show that upon exposure to drugs that depolymerize the mitotic spindle, or after genetic interference with tubulin, cells arrest in mitosis, but after approximately one hour leave the mitotic state to form a single undivided daughter cell, with unsegregated chromosomes.

This process is similar to a phenomenon earlier described in mammalian cells, called mitotic slippage, which refers to inability of mitotic cells to arrest indefinitely in the spindle checkpoint. In mammalian cells, this process had been shown to be related to slow destruction of cyclin B1, which eventually leads to mitotic exit. In the absence of a spindle, this coincides with failed cytokinesis, hence mitotic slippage leads to the formation of a 4N G1 cell.

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In principle these result are to be expected for cells that eventually leave mitosis in spindle poison to return to G1. However, in mammalian cells there is evidence that the spindle checkpoint is not inactivated in the process of mitotic slippage, whereas Figures 5 and 7 suggest in Aspergillus the checkpoint can be inactivated just before mitotic exit. Whereas the staining of kinetochore-bound Mad proteins, taken as a sole measure for an active checkpoint, is hard to decipher, especially Figure 7 seems to suggest Mad is lost from kinetochores even if cells in spindle drugs are inhibited from mitotic exit by an APC/C mutation.

We have addressed these concerns and feel that our data is now significantly clearer. See comments listed below for Figure 5 and 7.

This encompasses the main conceptual novelty of the paper, apart from a more detailed description of the adaptation to mitotic arrest in a filamentous fungus.

We feel that the fact that the SAC can be cyclically activated and then inactivated as cells transit multiple cell cycles without spindle function is also a conceptual novelty as pointed out by reviewer 2.

Further evidence to support the idea that the checkpoint can be switched off after 30-60 min in spindle drugs, is presented in Figure 3, where nucleolar reformation occurs (reportedly inhibited by the checkpoint), and in Figure 4 which shows that after 30 minutes in the arrest, cyclin B1 starts to be degraded more rapidly.

Altogether, this is taken as evidence that just before mitotic exit the spindle checkpoint is inactivated. The authors claim this may be a unique feature of a filamentous fungus, an organism which may experience exposure to environmental spindle toxins naturally, and so could adapt faster to such conditions.

We believe that it is likely that SAC inactivation without spindle assembly occurs in other organisms and suggest this in the discussion.

The presented data, although hard to decipher, confirm and provide further insight in the way cells can adapt to mitotic arrest and support some of the conclusions, but do not entirely prove that the checkpoint is really switched off. This is mainly due to the fact that the hard to measure kinetochore localization of Mad proteins is taken as the main read-out.

In the absence of biochemical data, what is missing is e.g. a proper quantitation of the effect of checkpoint inactivation, cyclin B1 destruction, in the experiment as shown in Figure 4B.

Is cyclin B1 destruction always seen as strictly biphasic, and are kinetics of cyclin B1 destruction in the last phase identical to that in normal cells? Are other APC/C substrates degraded at the right
time? How much cyclin B1 (%) is lost in the earlier, slippage phase?

See comments below for Figure 4.

Further, at present, given the large variation in the ways mammalian cells may respond to a sustained mitotic arrest, I feel the difference between Aspergillus and mammalian cells is exaggerated. It is yet insufficiently clear whether mammalian cells may also be able to eventually inactivate the checkpoint, later in the cell cycle.

We do discuss the possibility that a similar process might exist in certain mammalian cells in the last paragraph of the Discussion as indirect evidence suggests the SAC can be inactivated then reactivated when MT function is impaired.

We would like to point out that our findings are the FIRST to demonstrate that SAC inactivation can occur without spindle formation. We do not consider the argument that the potential of a similar, but not yet demonstrated, mechanism in other organisms to be a valid scientific reason to use to detract from the importance of our findings.

Therefore, it remains to be seen how different the two systems are. This should be more clearly emphasized in the beginning of the manuscript. It should also be pointed out better that the eventual mitosis without spindles is not normal and is only initiated after a considerable lag phase.

We have emphasized that SIME is occurring without DNA segregation and that this is clearly distinct from a normal mitosis. Importantly, however, when the SAC is inactivated, all other aspects of mitotic exit occur normally allowing efficient nuclear reformation and return to interphase.

Therefore, the inactivation represents an adaptation to sustained mitotic arrest in the checkpoint, which, biologically, might be highly related to the earlier described process of mitotic slippage. It is therefore unknown if this represents a unique feature of fungi or is a more common response in eukaryotes.

As mentioned above, we have included discussion about how other cell types likely respond to prolonged SAC activation by turning the SAC off.

Nevertheless, the observation that the checkpoint might be regulated independent of cyclin B1 destruction or mitotic exit, is conceptually interesting and should be further investigated.

Now, the authors only indicate that eventual checkpoint silencing occurs independent of protein synthesis (e.g. of a Cdk inhibitory protein). No attempts are taken to identify the proteins involved, although from other systems, various candidate proteins could be considered, which are not discussed here.

We have now considered and discussed candidate proteins in the Discussion. See comments for Figure 8.

Specific points:

Figure 1: Can the images be magnified and the intracellular features studied be more clearly indicated, in a drawing or with arrows?

We have magnified the images in Figure 1, indicated the periods of mitosis and indicated the position of the nucleus for the NES panel. The montage for Figure 1D has been replaced with a kymograph to make it directly comparable to those shown in Figure 1A.

Figure 2: Can the degree of chromatin (un)separation be shown more clearly?

For Figure 2 we have added an insert depicting the nucleus/nuclei at the end of each time course at higher magnification. We have also magnified each individual montage to show that only a
A single nucleus is generated following SIME.

Figure 4. See above, Figure 4B / Supp Figure 3 need to be extended, preferentially including additional APC/C substrates, and the cyclin B data further quantified and compared to controls.

We have quantified the levels of Cyclin B as suggested. We see only a minor difference in the kinetics of Cyclin B degradation during SIME compared to during exit from a normal mitosis. This is consistent with the SAC being inactivated at the end of the mitotic arrest which leads to APC/C activation and Cyclin B degradation. Quantitation is now included in Figure 4D.

We have now added new data demonstrating that Bub1, a known APC/C substrate, accumulates on kinetochores during an SAC arrest but is lost from kinetochores during exit from an SAC arrest without spindle function (Figure 5C).

Figure 5. Can Bub3 also be monitored? Are total fluorescent Mad2 levels similar? This should be included in this Figure.

We have monitored SAC kinase Bub1, a binding partner of Bub3. Like Mad1 and Mad2, Bub1 accumulates on kinetochores during SAC arrest but is completely removed from kinetochores as cells return to interphase during SIME. This is now included in Figure 5C and provides further evidence that the SAC is inactivated.

We do not see any significant changes in the total fluorescent levels of Mad2 when cells transit mitosis without spindle function. Note that Figure 5A has been replaced by a new series ñ see comments for Reviewer 2.

We have simplified Figure 5B by reducing the number of time points shown.

Figure 7. Can total Mad1 levels be included and Mad2 be shown here as well, as this Figure represents a major conclusion? Is the proteasome required for checkpoint silencing?

We have quantified the relative nuclear levels of Mad1 as now shown in Figure 7C. To obtain a clearer signal for Mad1, we have repeated the experiments in Figure 7A and B using strains containing Mad1-GFP together with Nup49-mCherry (instead of Mad1-mCherry together with Nup188-GFP) and obtained identical results.

To provide further evidence that the SAC is inactivated in these experiments we have added new data showing that Bub1 is also removed from kinetochores during a bimEAPC1 arrest without microtubule function.

The suggestion to look at the requirement for the proteasome is a good one which we had considered. We performed experiments using the MG115 proteasome inhibitor but found that it has no affect in A. nidulans. We think that, similar to budding yeast (Lee and Goldberg J. Biol Chem 1996), MG115 and similar peptide based inhibitors are not taken up by A. nidulans cells. However, our prediction would be that the SAC silencing would not require proteasome function. This is because the proteosome functions downstream of APC/C activation and our data indicate that the SAC can be inactivated without APC/C activation.

Figure 8. Could potentially involved factors be discussed, e.g. Cdc20 levels, any orthologs of p31-comet or Usp44? Could phosphorylation of SAC components be involved here, based on literature?

We agree that as Cdc20 is known to be synthesized during mitosis in human cells (Nilsson et al. NCB 2008), it potentially could be part of the mechanism. Unfortunately we have not been able to directly follow cdc20 as the GFP tagged version is not visible in A. nidulans, presumably because cdc20 is present at very low levels. However as our data suggest that SAC inactivation can occur without APC/C activity, we feel it unlikely that cdc20 is part of the mechanism.

p31 Comet orthologues are not present in fungi including budding yeast and A. nidulans.

There is not a clear orthologue of Usp44 in A. nidulans.
As requested by all three reviewers we have expanded our discussion of the potential mechanisms which regulate SAC inactivation without spindle function. This is now included in the Discussion on p17-18.

Figure 9: I am not convinced this represents a novelty, I would expect that upon wash-out of the drug, the next mitosis is normal.

We disagree that this is an expected result given that this nucleus has transited two interphases without nuclear division resulting in increased ploidy and extra spindle pole bodies. Further, this figure provides important evidence supporting our model that by inactivating the SAC when spindle assembly cannot occur, cells can return to interphase and subsequently undergo nuclear division when conditions are more favorable.

Referee #2 (Remarks to the Author):

This manuscript by De Souza and collaborators uncovers a new pathway involved in spindle checkpoint inactivation after a mitotic delay in Aspergillus nidulans. This finding has broad implications, including clinical relevance, and the quality of the data is impressive and obtained by direct observation in living cells. The authors do a great job characterizing mitotic exit events in this system and their dependence on checkpoint inactivation. They further show that this checkpoint inactivation mechanism surprisingly requires protein synthesis and is independent of Cdk1/cyclin B inactivation or mitotic exit. Most strikingly, the authors report that this checkpoint inactivation mechanism is reversible upon re-entry into mitosis over at least 3 polyploid cycles. The clear difference with the process of mitotic slippage in vertebrates, which apparently does not involve checkpoint inactivation, makes this a very interesting paper to be followed up. I have to say that the evidence that slippage in vertebrate mitotic cells does not require checkpoint inactivation is fairly weak, and it is based only on localization data of checkpoint proteins right after mitotic slippage, suggesting that despite their presence at kinetochores they may not be active. I am therefore highly enthusiastic for publication of the present study in EMBO J. I do however have a few concerns that would like to see addressed to meet the high standards for publication in EMBO J:

1- From the experiment described in figure 5A, although it is clear that Mad2 re-localizes to NPCs, it seems that some of it always remains associated with KT3s, as depicted with the Ndc80 marker. If true, this implies that, as it happens during mitotic slippage in vertebrates, Mad2 is still localized at KT3s during SIM, compromising the main conclusion of this paper. How could the authors rule out this possibility? For the sake of comparison, it would also be interesting to follow Mad2 localization during a normal mitotic exit.

This is a good point. During interphase, kinetochores locate in a single cluster adjacent to the nuclear envelope, while Mad1/Mad2 locate with nuclear pores at the nuclear envelope. By chance this can result in some Mad1/Mad2 locating near the interphase kinetochores in early G1. However, when images are taken at shorter time intervals we observe that Mad1 and Mad2 dissociate from kinetochores before re-accumulating in the nucleoplasm and then associating with nuclear pores. We have now replaced Figure 5A with an example showing loss of Mad2 from kinetochores followed by its nuclear re-accumulation and association with NPCs. This transition can also clearly be seen for Mad1 in Figure 5B. In addition, new data following Bub1 in Figure 5C clearly shows that it is completely removed from kinetochores.

As we have previously published the localization of Mad2 during a normal mitotic exit (De Souza et al. Mol. Biol. Cell. 2009; Supplementary data), we have not included it here.

2- Stating that cells undergoing SIM "complete all aspects of mitosis except for those which require spindle function", such as NUCLEAR DIVISION (=mitosis) is a counter sense to say the least. I strongly disagree about using the term SIM for "Spindle independent mitosis", since it is not indeed a type of mitosis because no nuclear division occurs. What about "spindle independent mitotic exit"?
We agree with the reviewer that spindle independent mitotic exit (SIME) is a better term than spindle independent mitosis (SIM). We have made the appropriate changes in the manuscript.

3- Although I don’t see it as a requirement for publication, taking an educated guess of possible candidates whose synthesis maybe required for SAC inactivation would have been a big plus.

We have done this, see response to reviewer 1, Figure 8.

4- I found intriguing that mitosis duration gradually decreases over each subsequent cycle in benomyl (fig. 6B). I think this is worth discussing in the text and explore some possible explanations. Could it be related with the dilution of a "time" factor, such as Mad2? In other words, how does the Mad2 overall pool change with ploidy?

We too found this consistent finding intriguing. One possibility is that it relates to the relative volume of the nucleus and cytoplasm. In the absence of microtubule function, cells undergo some polarized growth without nuclear division, decreasing the nuclear/cytoplasmic ratio. Conceivably this could decrease the duration of the SAC arrest, perhaps by dilution of a time factor as suggested. However, determining if this is the case is not straight forward and we feel it is out of the scope of this study.

5- One possibility to explain that protein synthesis is required for SAC inactivation is that it must counterbalance protein degradation (via the APC?). What happens if bimE7(APC1) cells at restrictive temperature and arrested in benomyl are treated with cycloheximide? Can the SAC now be inactivated with normal kinetics?

The idea that protein synthesis and degradation counterbalance each other is supported by recent work from the van Deursen lab (Malureanu et al., JCB 2010).

Our data suggest that the SAC is inactivated with normal timing during a bimE7APC1 arrest even though mitotic exit is prevented (Figure 7). However the suggested cycloheximide experiment is technically challenging and despite several attempts we have been unsuccessful. The main difficulty is that cycloheximide surprisingly prevents mitotic entry unless it is added less than ~1 minute prior to the end of G2. Thus only a very small percentage of cells enter mitosis following addition of cycloheximide. While adding cycloheximide after cells have already entered SAC arrest also delays SAC inactivation and mitotic exit (Figure 8C), the challenge of following the same field of cells before and after media exchange at 42°C has thus far prevented our success.

Minor remarks:

1- The SAC does not arrest mitosis until CORRECT bipolar attachments are established. The SAC will be satisfied even in the presence of some INCORRECT attachments, such as merotelic ones, as far as they are also bipolar. Please delete the word CORRECT from abstract and introduction.

These changes have been made.

2- The final statement in the introduction regarding mammalian cells is overly speculative and should be removed from this section.

This change has been made.

Referee #3 (Remarks to the Author):

This interesting paper documents clearly that Aspergillus cells blocked by the SAC will periodically escape, carry out at least some hallmarks of another cell cycle, then arrest again for a prolonged period, carrying out this cycle repeatedly and apparently with fairly regular timing.

The paper is largely descriptive beyond establishing this basic but interesting bit of biology, but the
description is interesting, especially in that the authors show that SAC components themselves exhibit hallmarks of cyclical SAC activation/inactivation as indicated by oscillating centromeric localization. It is shown that cyclin B proteolysis can be prevented without blocking at least one occurrence of SAC inactivation - it is not shown whether the repeated cycles do occur without cyclin B proteolysis, and this is interesting and important to know (see below). It is shown that protein synthesis is required for a relatively rapid SAC inactivation although mitotic exit apparently still occurs at longer times even in cycloheximide. This experiment suffers from the usual blunt-instrument problem with global inhibition of protein synthesis; while interesting, there is no clue as to what the critical synthesized protein(s) might be.

The supposition is made that these observations are somehow related to 'the' CDK-independent oscillator described by Orlando et al. This seems too simple to me - first, the Orlando work specifically describes a cyclin-independent transcriptional oscillator, so the specific hypothesis suggested could be tested (e.g. cell-cycle-regulated transcription proceeding at the same frequency as SAC inactivation in Aspergillus), rather than just brought up as a general speculation. Second, the Orlando work describes an oscillator functioning at zero mitotic cyclin (and probably not functioning at high mitotic cyclin, based on a lot of previous work), unlike the current putative SAC inactivator functioning at high mitotic cyclin. Third, there are at least two other cyclin-Cdk-independent oscillators described, one for centrosome/spindle pole duplication in fly and other embryos and in yeast, and one for release of the yeast Cdc14 phosphatase. At least the Cdc14 one clearly functions at high mitotic cyclin levels. So a more accurate statement would be that the SAC inactivation cycle could represent another oscillator, of unknown mechanistic relationship to the others previously characterized. This then leads to an important question (as clearly discussed in the Cdc14 oscillator work): how are the cyclin-Cdk cycle and the putative SAC inactivation cycle coordinated? Because if they are NOT coordinated the cell seems to run a risk of having the SAC inactivation cycle in an 'off' state just at the time when the checkpoint is needed, making the oscillator truly counterproductive. A 'good' biological design could be to have the cyclin-Cdk cycle and the SAC inactivation cycle entrained so that maximum sensitivity to the SAC would coordinate with the time when cyclin-Cdk is promoting spindle formation. Thinking about how such coordination of a putative SAC inactivation oscillator and the cyclin-Cdk oscillator might be achieved could be helpful in directing new experiments. For example, it would be a clue if the bimE mutation that allows SAC inactivation, nevertheless prevented SAC reactivation.

We thank this reviewer for their insightful suggestions. We have not seen SAC reactivation following its initial inactivation in our bimE<sup>del</sup> experiments.

While we agree with the comments regarding the potential transcriptional oscillator, we feel that determining if cell cycle regulated transcription is occurring in our system is beyond the scope of the current study. We have replaced our previous discussion about the potential of an oscillatory mechanism which leads to SAC inactivation as follows:

"One possibility is that the requirement for protein synthesis could represent a type of cell cycle oscillator which regulates the mitotic synthesis of one or more proteins. It remains to be seen if such an oscillator exists or if it is mechanistically related to other previously identified cell cycle oscillators (Ye et al, 1998; Orlando et al, 2008; McCleland and O'Farrell, 2008; Lu and Cross, 2010; Manzoni et al, 2010)."

Overall, I think this paper describes some very useful and intriguing initial observations, which while somewhat descriptive at present, have the possibility to lead to important new insights. Thinking more about how to place the observations in a broader context should suggest additional mechanistic and physiological experiments, that would broaden the significance and impact of the work.

We strongly feel that our manuscript goes well beyond initial observations and establishes important new insights into a topical area of research important to the cell cycle field and relevant to the development of cancer therapeutics.

By either chemically or genetically preventing spindle formation, we show that mitotically arrested cells inactivate the SAC when it cannot be satisfied. This has not been previously demonstrated. Using a genetic approach to prevent APC/C activation, we demonstrate that SAC inactivation can...
occur independently of mitotic exit. We then go on to place these findings in a physiological context. By many criteria, we show that when the SAC is inactivated without spindle formation, mitotic exit occurs normally even though nuclear segregation has failed. We not only show that such nuclei are functional, but demonstrate that they can continue through multiple cell cycles in which the SAC is remarkably reactivated each mitosis. Such cyclical activation-inactivation of the SAC without nuclear division is also completely novel and further demonstrates the SAC is turned off without mitotic spindles. Finally we show that mitotic protein synthesis plays an important role in regulating this process. While our findings also open up many new yet to be answered questions, this is the case for all new insights into biological processes.

Our manuscript is thus a significant scientific advancement with broad implications of interest to the readership of the EMBO Journal.

Thank you for submitting your revised manuscript for our consideration. All three original reviewers have now once more assessed it. While referee 2 has no more major concerns, referee 1 retains a number of (addressable) issues, while referee 3 is generally not satisfied with the way his/her points have been addressed. I am afraid I agree with this assessment, and I also note that you have not fully responded to what I raised in my revision letter.

One request was the identify or at least exhaustively discuss proteins whose synthesis or destruction may be involved in spindle-independent checkpoint inactivation and reactivation. I appreciate that you have added discussion on Cdc20 and cyclin expression during mitosis but other possible mechanisms especially synthesis of Cdk inhibitors should also be discussed further, as Sic1 synthesis in budding yeast can also cause mitotic exit. Furthermore following an addition suggestion of referee 1 I had asked you to investigate or at least comment on the potential involvement of NIMA kinase, which is microtubule bound and whose APC/C-independent destruction is required for mitotic exit - this is still outstanding.

The other requirement was to further comment on the major mechanistic implication of your current observations, the linkage to a possible underlying cell cycle oscillator system. You were asked to expand more on this but have instead chosen to deemphasize this part even more, which unfortunately makes the study a less-well suited candidate for The EMBO Journal now. I agree that a full elucidation of the links would go beyond the scope of the current submission, a disclaimer that I had included already in my original revision letter; however I strongly feel that the thoughtful and constructive input of referee 3 would have warranted more careful and scholarly discussions of the oscillator systems-aspect in the revised manuscript text. Especially the inclusion and discussion of the interesting results from the experiment proposed by referee 3, to look at possible bimE requirement for SAC reactivation after SIME, would have greatly benefitted the paper.

As I mentioned before, our normal policy is to allow only one major round of revision. In the current case, your paper could nevertheless become suitable with mostly textual revisions, better discussion and inclusion of currently excluded data as laid out above. I will therefore grant you an exceptional further round of revision here, should you be willing to address these remaining issues now, especially the request of referee 3 for the inclusion of the bimE mutant experiment and for a more thorough consideration of possible oscillator links along the lines mentioned in their original report. In addition, such a re-revision should also address referee 1’s remaining issues - including point 1 that asks for some validating of complete spindle depletion or alternatively for a qualification of the statements in the title.

It is my hope that you will be able to modify the manuscript accordingly, as I feel that all referees in principle considered your results as ‘descriptive’ in a positive and non-offensive way, yet were only hoping for the interesting observations to be fleshed out and discussed further with regard to their implications. In any case, I’d be happy to discuss these matters further with you should you desire additional clarifications.

Yours sincerely,
Referee #1 (Remarks to the Author):

This paper shows that, in mitosis of Aspergillus nidulans, spindle checkpoint proteins are eventually removed from the kinetochore cluster even after tubulin is genetically ablated or when cells are treated with a microtubule-depolymerizing drug. These cells degrade cyclin B and exit mitosis to form 4N G1 cells, which can continue to cycle.

From this, the authors conclude that the spindle checkpoint can, after a lag phase, be entirely satisfied in the absence of a mitotic spindle. This recovery process requires the synthesis of a protein that inactivates the checkpoint, or temporally removes spindle checkpoint proteins from the kinetochore cluster and activates the APC/C. Evidence is presented that release of spindle checkpoint proteins from the kinetochore cluster is not downstream of mitotic slippage or cyclin B1 destruction. The checkpoint is re-activated when undivided daughter cells enter a subsequent mitosis.

The authors paid attention to most of the points I raised and I think the current paper is improved as compared to the first version, both in terms of the experiments presented as well as in the way the data are interpreted. Nevertheless, I have several remaining questions.

1) In contrast to what the authors state in their rebuttal, it has been shown before that the spindle checkpoint can be satisfied in the presence of a spindle drug (Yang et al., J. of Cell Biology 186, pp675-684). In this paper it was shown that establishments of microtubule attachments to kinetochores can ultimately satisfy the checkpoint in Taxol-treated human epithelial cells.

In the present study, De Souza et al conclude that no mitotic spindles are formed at all, because a microtubule-depolymerizing drug has been used or one of the two beta-tubulin genes that exist in Aspergillus was genetically depleted. However, real evidence that no spindle with partial functionality remains at all after treatment with 2.4 microg/ml Benomyl OR that none of the remaining tubulins (tuba, tubb, tubc, or residual bna) might form a residual mitotic spindle, is not shown (Figure 1d, Sup.Figure S4).

Whereas the observation that the activity of the spindle checkpoint can cycle in the presence of effective concentrations of Benomyl is interesting, regardless of evidence that spindles are totally absent, the title of the current manuscript strongly indicates that no spindle are present at all.

I think these controls should thus either be shown, or the title must be changed into a more modest message (e.g. Cyclical Activation and Inactivation of the Spindle Assembly Checkpoint under Conditions of Spindle Perturbation). The Rieder study should be mentioned in the Introduction (e.g. p.4, end of first paragraph) and/or the Discussion section.

2) Figure 4D: the onset of cyclin B1 destruction is taken as t=0. Can the graph be plotted with a t=0 that is set at nuclear envelope breakdown?

3) Figure 5 and 7. A control for the total Mad1/Mad2/Bub1 levels is needed, e.g. as shown in Figure 6D for cyclin B (in 7c only the nuclear fraction of Mad1 is shown)? I asked this before, and the authors say in their rebuttal on page 3 that they 'do not see significant changes in the total fluorescent levels of Mad2' but these data are not shown in the paper.

4) page 17. Is it possible that an Aspergillus Cdk-inhibitor is produced during mitosis? This option is not discussed although there is some evidence in literature that high levels Cdk-inhibitors (which act independent of Cdk phosphorylation) can support mitotic exit, e.g. in yeast.
It looks to me that the cell shown in Figure 7D exits mitosis in the end, despite the bim inactivation. Can the authors comment on this? Mitotic exit in APC/C-inhibited cells could happen if cyclin-bound Cdk activity is inhibited. Could the authors indicate if APC/C-inhibited cells sometimes exit mitosis in the end without degrading cyclin B? Could this effect be sensitive to protein synthesis as well?

5) page 3, first paragraph: 'pseudo-metaphase'. I would prefer the term 'prolonged prometaphase'.

Referee #2 (Remarks to the Author):

In this revised manuscript De Souza and colleagues have addressed most of my major experimental concerns and significantly improved the discussion of the findings. I now find the paper suitable for publication and congratulate the authors for this very fine piece of work. My only request is to cite the following works when they refer and discuss the roles of protein synthesis for SIME:


The first paper was the first to show that cyclin B1 is even transcribed in mitosis and the second established the connection of cyclin B transcription and translation with sustained SAC response.

Referee #3 (Remarks to the Author):

In my review of this paper, I essentially predicted that the SAC cycles should stop after SAC inactivation in the presence of the bimE mutation, which the authors state in their response (but NOT in the revised MS) to be correct. I think this could be considered as an indication that the general issues that I brought up of coordination of the putative SAC oscillator and the CDK oscillator are relevant - otherwise, my prediction that bimE mutation might block SAC reactivation would have to come entirely from blue sky.

My overall comment on the paper was that it was largely descriptive but interesting, and would be improved by more thinking about oscillator coordination, with related experimentation on what manipulations blocked the SAC cycle in different phases. Since in my review I specified such an experiment with bimE, and correctly predicted the result, it is surprising to me to find the revision with even less discussion of these issues, and the result that I predicted not mentioned in the text although it is described in the response to reviewers.

So effectively I think the paper is unchanged with respect to the issues I raised. Perhaps the authors wish to reserve the very interesting bimE result for a fuller later analysis, in which case the paper is intentionally kept at what I still consider a relatively initial and descriptive level (although the authors, as is their right, take exception to this characterization). In my opinion, placing the work in a quantitative, systems-level analytical context is entirely the right approach, especially since at this point it requires little more than citing observations already made and thinking about the system in a more analytical way.

2nd Revision - authors' response 05 May 2011

We thank you for giving us the opportunity to resubmit our manuscript to the EMBO Journal. We have carefully considered the suggestions and comments of the reviewers and modified our manuscript accordingly. In response to reviewer 1 we have quantitated the absence of mitotic spindles and also changed the title of the manuscript to: “Regulated Inactivation of the Spindle Assembly Checkpoint without Functional Mitotic Spindles”. We have also included quantification
of protein levels as requested for all the relevant figures. In the revised version we have discussed why we think inactivation of Cdk1-Cyclin B by the synthesis of Cdk inhibitors is unlikely to contribute to SAC inactivation. In response to reviewer 3 we have now included data (Supplemental Figure S9) demonstrating that the SAC is not reactivated during a bimE7APC1 mitotic arrest without spindle function. These data have also been discussed in relation to the potential that SAC activation-inactivation is regulated by entrained cell cycle oscillatory systems as requested by reviewer 3.

Following a suggestion from Reviewer 1 you had asked that we “look at the potential involvement of NIMA, as its APC/C-independent destruction is also required for mitotic exit and as the normal microtubule binding of NIMA would be perturbed in your experimental setup (it might therefore be worth to also look if microtubule-stabilizing drugs would have similar effects or not).” However, we have published that NIMA destruction occurs in an APC/C dependent manner (Ye et al., 1998; Mol Biol Cell 9, 3019-3030) and we are not aware of any evidence that NIMA is bound to microtubules. At this time we have no data, or other reason, that might suggest NIMA is playing a role in inactivation of the SAC without spindle functions. The detailed changes to our manuscript and new experimental data are outlined in our response to each reviewer.

Referee #1 (Remarks to the Author):

This paper shows that, in mitosis of Aspergillus nidulans, spindle checkpoint proteins are eventually removed from the kinetochore cluster even after tubulin is genetically ablated or when cells are treated with a microtubule-depolymerizing drug. These cells degrade cyclin B and exit mitosis to form 4N G1 cells, which can continue to cycle. From this, the authors conclude that the spindle checkpoint can, after a lag phase, be entirely satisfied in the absence of a mitotic spindle. This recovery process requires the synthesis of a protein that inactivates the checkpoint, or temporally removes spindle checkpoint proteins from the kinetochore cluster and activates the APC/C. Evidence is presented that release of spindle checkpoint proteins from the kinetochore cluster is not downstream of mitotic slippage or cyclin B1 destruction. The checkpoint is re-activated when undivided daughter cells enter a subsequent mitosis.

The authors paid attention to most of the points I raised and I think the current paper is improved as compared to the first version, both in terms of the experiments presented as well as in the way the data are interpreted. Nevertheless, I have several remaining questions.

1) In contrast to what the authors state in their rebuttal, it has been shown before that the spindle checkpoint can be satisfied in the presence of a spindle drug (Yang et al., J. of Cell Biology 186, pp675-684). In this paper it was shown that establishments of microtubule attachments to kinetochores can ultimately satisfy the checkpoint in Taxol-treated human epithelial cells. We had shown by following a GFP tagged version of the -tubulin (tubA) that no mitotic spindle formation occurs in the presence of 2.4 ug/ml benomyl and that none of the remaining tubulins (tuba, tubb, tubc or residual bene) might form a residual mitotic spindle, is not shown (Figure 1d, Sup. Figure S4). We had shown by following a GFP tagged version of the -tubulin (tubA) that no mitotic spindle formation occurs in the presence of 2.4 ug/ml benomyl (Figure 9B). To further emphasize this important finding we have changed the last sentence of the first paragraph on P7, including quantitation, which now reads:

"Examination of GFP- -tubulin confirmed that mitotic spindles did not form when benomyl treated cells transited mitosis (6 independent experiments, n = 27 cells, eg. Figure 9B) as also evidenced by the failure of nuclear division."
Whereas the observation that the activity of the spindle checkpoint can cycle in the presence of effective concentrations of Benomyl is interesting, regardless of evidence that spindles are totally absent, the title of the current manuscript strongly indicates that no spindle are present at all. I think these controls should thus either be shown, or the title must be changed into a more modest message (e.g. Cyclical Activation and Inactivation of the Spindle Assembly Checkpoint under Conditions of Spindle Perturbation). The Rieder study should be mentioned in the Introduction (e.g. p.4, end of first paragraph) and/or the Discussion section.

We have changed the title to:
"REGULATED INACTIVATION OF THE SPINDLE ASSEMBLY CHECKPOINT WITHOUT FUNCTIONAL MITOTIC SPINDLES"

We feel that collectively our data provide compelling evidence that the SAC can be inactivated in the absence of functional mitotic spindles. We have confirmed that mitotic spindle formation does not occur when cells transit a mitotic SAC arrest in the presence of 2.4 ug/ml benomyl. Further, genetic deletion of an essential -tubulin gene gives an identical transient mitotic SAC arrest in the absence of any drug treatment. Together with the fact that cells exit the mitotic SAC arrest in the absence of DNA division we feel this provides compelling evidence that the SAC can be inactivated without functional mitotic spindles.

We have now included discussion of the Yang/Rieder reference at the end of the first paragraph on p 4:
"For example, when microtubules are stabilized by taxol mitotic exit occurs when syntelic kinetochore attachments become stabilized in a manner which leads to SAC satisfaction (Yang et al, 2009)."

2) Figure 4D: the onset of cyclin B1 destruction is taken as t=0. Can the graph be plotted with a t=0 that is set at nuclear envelope breakdown?

We have now included the full time course for the experiments shown in Figure 4D. Mitotic entry occurs following t=0 and is defined as when Cyclin B is partially released to the cytoplasm resulting in a decrease in its nuclear levels.

3) Figure 5 and 7. A control for the total Mad1/Mad2/Bub1 levels is needed, e.g. as shown in Figure 6D for cyclin B (in 7c only the nuclear fraction of Mad1 is shown)? I asked this before, and the authors say in their rebuttal on page 3 that they 'do not see significant changes in the total fluorescent levels of Mad2' but these data are not shown in the paper.

We have now included data showing the total cellular levels of Mad1, Mad2 and Bub1 during transit through SAC arrest in Figure 5.
For Figure 7, we have added data to show both the nuclear and total cellular levels of Mad1/Bub1 during each time course.

4) page 17. Is it possible that an Aspergillus Cdk-inhibitor is produced during mitosis? This option is not discussed although there is some evidence in literature that high levels Cdk-inhibitors (which act independent of Cdk phosphorylation) can support mitotic exit, e.g. in yeast.

We do not think that this is the case as our APC/C inactivation experiments indicate that the SAC can be inactivated without mitotic exit under conditions in which Cdk1/Cyclin B activity remains elevated. We have now added the following to the discussion:
"In addition, as Cdk1/Cyclin B activity remains elevated when BIMEAPC1 is inactivated (Ye et al, 1998), these experiments strongly suggest that Cdk1/Cyclin B inactivation by accumulation of a Cdk1 inhibitory protein or by inhibitory Cdk1 tyrosine phosphorylation is not leading to SAC inactivation without spindle function."

It looks to me that the cell shown in Figure 7D exits mitosis in the end, despite the bim inactivation. Can the authors comment on this?

The data presented to not support that the cell in Figure 7D (now Figure 7E) has exited mitosis.
During bimE7 APC1 arrest, NPCs remain disassembled and Cyclin B is stabilized indicating that cells are arrested in mitosis. We think that perhaps the reviewer is referring to the second, smaller, focus of Cyclin B that appears at the end of the time course (Figure 7E). The appearance of this second focus of Cyclin B is a result of the increase in Cyclin B levels which occur during a bimE7 arrest. This accumulation of Cyclin B has simply made a weak focus of Cyclin B stronger. Such weak Cyclin B foci are often present during an SAC arrest in addition to the main focus of Cyclin B in the SPB/kinetochore region (eg Figure 4A).

Mitotic exit in APC/C-inhibited cells could happen if cyclin-bound Cdk activity is inhibited. Could the authors indicate if APC/C-inhibited cells sometimes exit mitosis in the end without degrading cyclin B? Could this effect be sensitive to protein synthesis as well?

Based on our experiments following NPC reformation (as a marker for mitotic exit) and Cyclin B, we do not think this occurs during a bimE7APC1 mitotic arrest.

5) page 3, first paragraph: 'pseudo-metaphase'. I would prefer the term 'prolonged prometaphase'. We have made this change.

Referee #2 (Remarks to the Author):

In this revised manuscript De Souza and colleagues have addressed most of my major experimental concerns and significantly improved the discussion of the findings. I now find the paper suitable for publication and congratulate the authors for this very fine piece of work. My only request is to cite the following works when they refer and discuss the roles of protein synthesis for SIME:


The first paper was the first to show that cyclin B1 is even transcribed in mitosis and the second established the connection of cyclin B transcription and translation with sustained SAC response.

We have added these references as requested.

Referee #3 (Remarks to the Author):

In my review of this paper, I essentially predicted that the SAC cycles should stop after SAC inactivation in the presence of the bimE mutation, which the authors state in their response (but NOT in the revised MS) to be correct. I think this could be considered as an indication that the general issues that I brought up of coordination of the putative SAC oscillator and the CDK oscillator are relevant - otherwise, my prediction that bimE mutation might block SAC reactivation would have to come entirely from blue sky.

My overall comment on the paper was that it was largely descriptive but interesting, and would be improved by more thinking about oscillator coordination, with related experimentation on what manipulations blocked the SAC cycle in different phases. Since in my review I specified such an experiment with bimE, and correctly predicted the result, it is surprising to me to find the revision with even less discussion of these issues, and the result that I predicted not mentioned in the text although it is described in the response to reviewers.

So effectively I think the paper is unchanged with respect to the issues I raised. Perhaps the authors wish to reserve the very interesting bimE result for a fuller later analysis, in which case the paper is intentionally kept at what I still consider a relatively initial and descriptive level (although the authors, as is their right, take exception to this characterization). In my opinion, placing the work in a quantitative, systems-level analytical context is entirely the right approach, especially since at this
point it requires little more than citing observations already made and thinking about the system in a more analytical way.

We have now included data showing that cells which remain arrested in a bimE7APC1 mitotic arrest do not reactivate the SAC in Supplemental Figure S9. We have extended our discussion on the potential that SAC inactivation is regulated by a cell cycle oscillatory mechanism P18-19 as follows:

"Inactivation of the SAC without spindle function leads to cell cycle oscillations without mitotic division. In A. nidulans, similar oscillations have previously been uncovered upon rapid inactivation of the APC/C using the bimA1APC3 temperature sensitive allele (Ye et al, 1998). It was hypothesized that the bimA1APC3 mutation rendered the APC/C less responsive to the accumulation of proteins which are both involved in APC/C activation and also APC/C substrates. Following rapid inactivation of bimA1APC3, this then causes delay in a mitotic state while such proteins accumulate to a level that activates the mutant APC/C to allow mitotic exit and repetition of the process (Ye et al, 1998). A similar scenario could be in play regarding the cyclic SAC activation-inactivation reported here but with an undefined protein(s) accumulating to a critical level during SAC arrest to trigger SAC inactivation and exit from mitosis into a new cell cycle. The extended SAC arrest observed when protein synthesis is perturbed is consistent with such a model.

Insights to how the SAC activation-inactivation cycle might be regulated and integrated with other cyclic cell cycle regulatory systems come from several experimental systems in which cell cycle oscillations can be uncoupled from cell cycle progression (Haase et al, 2001; Orlando et al, 2008; McCleland and O'Farrell, 2008; Lu and Cross, 2010; Manzoni et al, 2010). These findings have led to the concept that Cdk1-Cyclin acts as a master oscillator which entrains a series of peripheral oscillators that control individual cell cycle events (Lu and Cross, 2010). Thus the SAC activation-inactivation cycle might be a peripheral oscillator that is entrained to occur at the appropriate times by the Cdk1-Cyclin oscillator. It is known that peripheral oscillators can also feedback on the CDK-Cyclin oscillator resulting in mutual entrainment (Lu and Cross, 2010). This would be highly likely for a potential SAC regulatory oscillator because SAC inactivation leads to Cyclin B degradation allowing cells to transit interphase and reactivate the SAC when they enter the subsequent mitosis. Such a potential mechanism could explain how the cycles of SAC activation and inactivation that occur without spindle function become coupled with the cell cycle. Supporting this idea, preventing Cyclin B degradation and mitotic exit by inactivating the APC/C is not required to inactivate the SAC, but is required for reactivation of the SAC during a prolonged mitotic arrest without spindle function (Supplementary Figure S9). As we discuss below, such a system which allows cells to transit multiple cell cycles without normal spindle function might act as a survival mechanism in A. nidulans."

Acceptance letter 09 May 2011

Thank you for submitting your re-revised manuscript for our consideration. I have now had a chance to look through it and to assess your responses to the comments raised by the reviewers, and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

You shall receive a formal letter of acceptance shortly.

Yours sincerely,

Editor
The EMBO Journal