THE
EMBO
JOURNAL

Manuscript EMBO-2016-94831

Dbf4-dependent kinase (DDK) and the Rtt107 scaffold promote Mus81-Mms4 resolvase activation during mitosis

Lissa Princz, Philipp Wild, Julia Bittmann, Fransisco Aguado, Miguel González Blanco, Joao Matos and Boris Pfander

Corresponding author: Boris Pfander, Max-Planck Institute of Biochemistry

Review timeline:

Submission date:	22 May 2016
Editorial Decision:	20 June 2016
Revision received:	15 November 2016
Editorial Decision:	06 December 2016
Revision received:	15 December 2016
Accepted:	19 December 2016

Editor: Hartmut Vodermaier

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 20 June 2016

Thank you for submitting your manuscript on DDK roles in Mus81 regulation for our consideration. We have now received feedback from three expert referees, whose reports you will find copied below. The referees consider the topic as well as your main findings interesting in principle and therefore potentially suitable for The EMBO Journal, however they also raise a significant number of major concerns that would need to be satisfactorily addressed before publication may be warranted.

Given the mainly technical/experimental nature of the referees' criticisms, I would like to give you an opportunity to address them via a major revision of this study. We might further arrange for an extended revision period, during which time the publication of any competing work elsewhere would as usual have no negative impact on our final assessment of your own study.

REFEREE REPORTS

Referee #1:

This manuscript presents a detailed study on the role of Dbf4-dependent kinase (DDK) in Mus81-Mms4 regulation. By mass-spectrometry and pull-down analysis, the authors first identify that Cdc7 and Dbf4 as specific interactors of Mms4. They show that DDK can phosphorylate Mms4 and that

DDK and Cdc5 target Mus81-Mms4 in an interdependent manner. The authors further present data that DDK-mediated phosphorylation of Mms4 is required for Mus81 activation during mitosis. Finally, the authors show that Rtt107 scaffold protein, but not Dpb11 and Slx4, interacts with Cdc7 and is required to target DDK and Cdc5 to the complex to enhance Mus81-Mms4 activity in mitosis.

These findings provide the first evidence that a third cell cycle kinase, Cdc7-Dbf4 (DDK), together with the Rtt107 scaffold protein, is involved in regulating Mus81-Mms4 activation during mitosis. Although detailed roles of these three kinases are not clear, but the interplays of multiple kinases and its functional interaction with a scaffold protein provides a novel aspects on mechanisms that exert timely and spatially regulated chromosome transactions. The results extend the understanding of the regulatory framework that controls cell cycle-regulated DNA joint molecule (JM) resolution and therefore it is likely to be of broad interest in the field.

The data presented are of high quality and convincing. However, the interpretation of the data is a little confusing. There are a lot of arrows in the Figure 7d model, but it is not very clear how the three kinases and Rtt7 contributes to the regulation of Mms4.

Given the previous reports on Cdc7 phosphorylation reactions, it appears that Cdk would be a priming kinase which facilitates the Cdc7 mediated phosphorylation of the neighboring serine residues. This would create the target of the polobox of Cdc5 kinase, recruiting Cdc5 to Mms4. This step may be facilitated by the N-terminal segment of Dbf4 which Cdc5 can interacts with. Stable binding of Cdc7 to Mms4 may be facilitated by the presence of Rtt107. However it appears that phosphorylation of Mms4 does not appear to be that much affected (as judged by the mobility shift of Mms4 in Figure 6b). SILAC analyses in Figure 7a also show that Cdc5-mediated phosphorylation seems to be affected but not the (S/T)(S/T) phosphorylation by Cdc7. Thus, Rtt007 may stabilize the interaction between Cdc7 and Mms4 or facilitates the recruitment of Cdc7, but the phosphorylation of Mms4 by Cdk-Cdc7 itself may not be that much affected in rtt107Δ mutant. However, due to instability of Cdc7 on Mms4, Cdc5 may not be efficiently recruited to Mms4, leading to loss of Cdc5-mediated phosphorylation.
On the other hand, reduction of Mms4-Rtt107 interaction in cdc7Δ or cdc5as indicates phosphorylation of Mms4 by Cdc7-Cdc5 may even further stabilize the Mms4-Rtt107 interaction.

With these models in mind, I present some comments and questions below. I hope the authors can present clearer models (not necessarily the one above; multiple models could be possible) that could be evaluated by future experiments.

Major comments

Figure 1b
What is the effect of DDK and CDK+DDK on Cdc5-mediated phosphorylation of Mms4? It is predicted that the dual phosphorylation of SS/ST would induced the binding of Cdc5 through its polo box and stimulate the phosphorylation of Mms4 by Cdc5.

Figure 3a and figure S3a; Figure 7a and Figure S7a
Why are there two sets of SILAC data?

Figure 4b MMS4A-8A mutant does not interact with Cdc5 at all. This suggests that (S/T)(S/T) phosphorylation is required for Cdc5 recruitment. Reduced interaction of Cdc7 with the same mutant is surprising. Is Rtt107 bound to MMS4-8A as efficiently as the wild-type Mms4? Above hypothesis predicts that MMS4-8A may interact with Rtt107 less stably.

Figure 4a and 4c and Figure 7b
The level of Mms4 has been checked. However, authors also need to examine whether association with Mus81 is affected or not.

The activity of MMS4-8A is weaker than that of the wild type but there is still significant activity in the mutant. This raises the question that Cdc7-mediated phosphorylation really regulates the enzymatic activity.
Two approaches can be taken to answer this.
Pretreat the purified Mms4 proteins with combinations of kinases and examine the effect on its

activity. Alternatively, create the DE substitution of the putative phosphorylation sites (phosphomimic mutants) and examine the enzymatic activity.

Does Cdc5 play any role in the enzymatic activity of Mus81-Mms4? What is the enzymatic activity of Mms4 in cdc5as mutant?

In rtt107Δ cells, the enzymatic activity is reduced, but not to the extent of cdc7Δ. Is it due to the absence of co-immunoprecipitated Rtt107? Is Rtt107 present in the immunoprecipatated fraction used for the assays? Does the presence of Rtt107 affect the enzymatic activity of Mms4? If that is the case, interpretation of these biochemical data becomes complicated.

JM resolution in vivo
It will provide stronger evidence if the authors can present data that show the JM structures cannot be resolved in cdc7Δ, mms4-8A, rtt107Δ mutants in vivo (using 2D gel).

Minor comments:

Page 3, subtitle: the word 'target' has extra 'e'.

Figure 3b
I apologize if I am mistaken, but is the left-right switched on this chart?
I thought Cdc7 target phosphorylation decrease in the cdc7Δ cells. That means the red dots should be on the right side?

Figure 4f and 7c: it will be easier to understand the figures if the label is written in CO/NCO instead of the word red, red/white and white.

Page 7, figure S3c was mentioned but should be S3b.

Figure 5b, 5d
Dots on lane 3 and 6 are not necessary?

Figure 6a
Does Cdc5 interact with Rtt107?
Why is there no interaction between Dbf4 and Rtt107?

Figure 6c
Are Cdc7 and Dbf4 present in the RTT107 IP?

Figure S2d
The shift is not very clear. I recommend the use of phostag gel to show the difference more clearly.

Figure S5b
There are three bands for Slx4 in the input, while only one band is co-immunoprecipitated. Is there any explanation for this?

Referee #2:

Recent studies have demonstrated that Cdk1 and Cdc5-mediated phosphorylation of Mms4 activate the structure-specific endonuclease (SSE) Mus81-Mms4 in budding yeast. This ensures maximal activity of Mus81-Mms4 in G2/M when cells need to efficiently resolve potential DNA joint molecules (JM) before anaphase to allow faithful chromosomal segregation.

In the present study, Pfander and colleagues identify a new layer of regulation of Mus81-Mms4 showing that it also relies on the Cdc7-Dbf4 kinase (Dbf4-dependent kinase, DDK).
After identifying DDK as a new partner of Mms4 by mass spectrometry analysis, the authors move on to show that it can hyperphosphorylate Mms4 in vitro. Using Mms4 peptides, they convincingly

show that DDK phosphorylates (S/T)(S/T) motifs on the first (S/T), only when the second (S/T) residue is already phosphorylated (likely by Cdk1).

In agreement with the in vitro results, DDK is next shown to interact and to contribute to phosphorylation of Mms4 in mitosis. Interestingly, it appears that DDK and Cdc5 interact with and phosphorylate Mms4 in an interdependent manner. Their interaction with Mms4 also strongly relies on the activity of Cdc28. Consistent with a contribution of DDK in the catalytic activation of Mus81-Eme1, processing of a nicked HJ by Mus81-Mms4 partially purified from mitotic cells is greatly reduced when pulled-out from DDK mutants.

Using mass spectrometry analyses, the authors identify putative DDK-dependent phosphorylation sites (mainly in "SS" sites) and engineer an mms4-8A mutant in which the first Serine in the SS motifs is mutated to Alanine. This mutant displays a hypomorphic phenotype. In contrast to the mms4Δ mutant, it is not hypersensitive to MMS nor synthetic lethal with sgs1Δ but the mms4-8A sgs1Δ double mutant displays a growth defect and is more sensitive to MMS compared to the sgs1Δ single mutant.

Mus81-Mms4-8A partially purified from mitotic cells has reduced catalytic activity compared to the WT complex but not as reduced as that of a complex pulled from DDK mutants. Importantly, deleting the N-terminal 109 residues of Dbf4, which removes a previously identified Cdc5-binding site, results in reduced phosphorylation of Mms4 and mirrors the phenotypes of the mms4-8A mutant. This strongly suggests that DDK-dependent phosphorylation of Mms4 is required for full activation of Mus81-Mms4 in mitosis.

Having previously published that the scaffold protein Dbp11 interacts with phosphorylated Mms4 and Slx4, the authors tested whether Dpb11 may be required for the DDK and Cdc5-dependent activation of Mus81-Mms4 in mitosis. For this, they identify the Dbp11-interacting region in Mms4 and engineer an Mms4-S201A mutant deficient in Dbp11 binding. Importantly, the Mms4-S201A mutant still interacts with DDK and Cdc5 and is at least as active as the WT complex on nicked HJs when partially purified from mitotic cells. These results strongly suggest that Dpb11 is not required for the DDK and Cdc5-dependent phosphorylation of Mms4 and activation of Mus81-Mms4 in mitosis.
Finally, the authors investigate whether Rtt107 may contribute to the recruitment of DDK and Cdc5 to Mus81-Mms4. Using Y2H they show that Rtt107 binds Cdc7. While Slx4 is known to bridge Rtt107 and Dpb11 and Dpb11 to bridge Mms4 and Slx4, the authors unexpectedly find that neither Slx4 nor Dpb11 are required for Rtt107 to co-purify with Mms4, suggesting that Rtt107 may also directly associate with Mms4.

While at first glance (phospho-shift) Rtt107Δ cells display normal Mms4 hyperphosphorylation, differential proteomics reveal a decrease in Cdc5-dependent phosphorylation sites in Mms4 in absence of Rtt107. In agreement, Mus81-Mms4 partially purified from Rtt107Δ cells displays a reduced activity on nHJs in vitro.

This is a really exciting paper that brings new insight into the control of the Mus81-Mms4 endonuclease by adding a new layer of regulation based on the unanticipated Rtt107-regulated cooperation between the DDK and Cdc5 kinases in mitosis.
The data seem overall solid and these findings should be of interest to a broad readership. However, a few points listed below need to be clarified.

Q1:
Fig1C: Why does peptide 2 which should be di-phosphorylated run faster than monophosphorylated peptide 1? The migration profiles are more coherent for peptides 4 and 5, but what happens with peptide 6, which should run at the same position as the monophosphorylated peptide 5 once it is further phosphorylated by DDK?

Q2:
Fig2B: Why is Mms4 still heavily phosphorylated when Cdc28 is inhibited? Shouldn't Mms4 no longer be phosphorylated in absence of Cdc28 activity and association with Cdc5 and DDK?

Q3:
Fig2C: Why when the Cdc5 kinase activity is completely inhibited, Mms4 is much less phosphorylated than in Fig2c even though there are detectable levels of Cdc5 and Cdc7 that associate with Mms4?

Q4:
Fig 2D: Deleting Cdc7 abolishes the Cdc5-Mms4 interaction but Mms4 still seems to phosphorylated?

Q5:
Fig S2E: The autors claim that "forced expression of Cdc5 in S-phase leads to the premature occurrence of Mms4 hyperphosphorylation in S-phase".
Although Cdc5 may indeed be the rate-limiting factor for hyperphosphorylation of Mms4, the data provided in Fig S2E don't strongly support this. Indeed in condition #6 when Cdc5 is over-expressed in S-phase cells to levels comparable to those in G2/M (conditions #4 and 7), there is only a small level of phosphorylation of Mms4 compared to conditions 4 and 7. Furthermore, FACS profile show that in condition 6 a fraction of cells are already in G2/M, which may account for the Mms4 phosphorylation.

Q6: The data presented in Fig 6D show that the Mms4-S201A mutant, while deficient in binding to Dpb11, is perfectly proficient in interacting with Slx4. Furthermore, Fig S6 shows that loss of Rtt107 strongly impairs binding of Slx4, but not of Dpb11, to Mms4. These results suggest that there is no relationship between Dpb11 and Slx4 in binding to Mms4. Rather than the previously proposed role for Dpb11 in bridging Slx4 and Mms4 in mitosis (Gritenaite 2014), the current study suggests that it is Rtt107 that allows the mitotic interaction of Slx4 and Mms4. Could the authors discuss this further in light of their previously published study?

Q7: The authors state that DDK is not required for binding of Mms4 to Slx4 and Dpb11. However, in Fig 2E there seems to be some decrease (although modest) in the amounts of Slx4 and Dpb11 that co-purify with Mms4 in Cdc7Δ cells. Could the authors please clarify this point?

Q8:
Fig5: The authors claim that there is no effect of the S201A mutation on hyperphosphorylation of Mms4. This seems to be indeed the case in Fig 5B and 5D. However, phosphorylation of Mms4 S201A is reduced in Fig 5E. Moreover, regarding the HJ resolution assay with S201A, it is much more efficient in Fig 5E than in Fig 4 and Fig 7. To unambiguously claim that the S201A mutation has no effect on Mus81-Mms4 activity, this would need an in vitro nuclease assay with kinetics (and efficiency) similar to Fig 4 and Fig 7.

Q9:
Why does deletion of Rtt107 only affect phosphorylation of Mms4 mediated by Cdc5 and not phosphorylation by DDK? Similarly, why does DDK deficiency not really affect Cdc5-mediated phosphorylations? Although it is clear that there is interdependency between DDK and Cdc5 for binding to Mms4, it seems that each kinase can still phosphorylate, at least partially, Mms4.

Q10 (related to Q8):
This is an overall comment on the in vitro nuclease assays. Apart from data in Fig 5, the efficiency of processing of a nHJ seems surprisingly low given the fact that this is a structure that gets very efficiently processed even by recombinant Mus81-Mms4. What are the differences between reactions in Fig 5 and those shown in Fig 4 and 7?
Are we looking here at the fully activated Mus81-Mms4 complex? Have the authors assessed the contribution of DDK to the stimulation of Mus81-Mms4 on other structures? What is the activity like of Mus81-Mms4 from S-phase cells? Based on previously published work by Tercero and colleagues it is expected to be marginal. If so, what impact does over-expression of Cdc5 in S-phase cells have on the activity of Mus81-Mms4?

Minor:

Page 3: Mus81-Mms4 is a target (not "targete")

Page 7: (See Fig S3b) (not S3C)
Page 12: "dbf4-ΔN109 .... shows a strinkingly similar MMS hypersensitivity as the phosphorylation site mutant mms4-8A"
→ Add "in the absence of Sgs1" because single mutants do not display MMS hypersensitivity

Referee #3:

The article by Princz et al. describes studies in yeast on the role of kinases and scaffolding proteins in the activation of the DNA structure-selective endonuclease, Mus81-Mms4. In particular, they focus on its activation in mitosis because previous studies have shown that this nuclease is activated by phosphorylation in yeast and human mitotic cells. The subject matter is certainly suitable for EMBO J., but the study has several weaknesses that diminish my enthusiasm.

Specific Comments

1) The data in Figures 1b/1c/2 do not appear to me to be internally consistent. The authors argue due to data in Figure 1c that CDK primes phosphorylation by DDK, but the evidence in Figure 1b does not support this. In Figure 2, it is shown that phosphorylation of Mms4 is abolished in cdc7 and cdc5 deficient stains - but not in cdk1 deficient cells. Moreover, in vitro kinase assays like the one shown in Figure 1b are very hard to interpret because kinases tend to phosphorylate any protein given to them. Some measure of specificity is essential.

2) In Figure 2a the FACs data from Suppl. Figure 2a should be included and the time points indicated on the figure. Putting a series of dots over the lanes tells the reader nothing.

3) Why is the Flag input not shown in all cases in Figure 2? It should be.

4) There is a consistent issue with the authors providing very little experimental detail in the manuscript body and Figure legends. For example, were the cells in Figure 2b/c/d/e arrested with nocodazole?

5) Scc1 is shown as a canonical Cdc5 substrate in Suppl. Figure 2d, but the blot is unconvincing.

6) In Figure 2b, inactivation of Cdk1 has only a slight effect (if any) on Mms4 phosphorylation, but abolishes Cdc5 and Cdc7 association. This is not what is described in the text.

7) The biochemistry described in Figures 4 and 7 is very weak. There are 2 major issues. First, the incubation timers are very long, indicative of very weak nuclease activity. Second, the product bands are so faint - as to be nearly impossible to interpret. In particular, in Figure 7, I would doubt if the activity is altered much, if at all. These experiments need to be repeated to get decent levels of cleavage (> 50%). Also, it is not acceptable to plot the graphs with the vertical axis not beginning at zero. This is done presumably to try to make any tiny difference look impressive.

8) In Figure 4, why was residue 286 not mutated? It looks like the best candidate to my eye?

9) Figures 5b and 5d could be combined.

1st Revision - authors' response	15 November 2016

Report continued on next page.

Point-by-point response (Princz et al.) – EMBOJ-2016-94831

Reviewer 1

We are very encouraged by the very positive response of this reviewer, who praises the novelty of our study and finds that "the presented data are of high quality and convincing." In particular we would like to thank this reviewer for the constructive criticism towards our paper. The points he/she has raised were very interesting and we addressed most of them experimentally. This includes additional controls, but also completely new lines of experimentation, such as new phosphorylation site analysis by MS using overexpression of Mus81-Mms4, a new *mms4* mutant, several new in vitro kinase, JM resolution and IP experiments.
Moreover, the reviewer mentioned a small number of points, where he/she found our initial presentation unclear. We have clarified these points in the revised version of our paper, which in our view improved a lot in the process. Finally, we would like to thank this reviewer for paying particular attention to the molecular interplay within the kinase cascade. In the revised version of our manuscript we have put a specific focus on the interplay of the kinases and Rtt107. Indeed we think that our data was best explained if Rtt107 was involved in an amplification mechanism that bears high resemblance to kinase priming. We have also rewritten the discussion and hope that the reviewer will find the model much clearer, now.

Specific points:
(1) *"Figure 1b: What is the effect of DDK and CDK+DDK on Cdc5-mediated phosphorylation of Mms4? It is predicted that the dual phosphorylation of SS/ST would induced the binding of Cdc5 through its polo box and stimulate the phosphorylation of Mms4 by Cdc5."*

Indeed, we also thought that the SS/ST motifs in Mms4 might - after a priming phosphorylation - bind to Cdc5 via the polo box domain (PBD) and thus stimulate Mms4 phosphorylation by Cdc5. However, to our knowledge and in contrast to the reviewers suggestion, the more likely scenario was that a single not dual phosphorylation of the SS/ST motif would be involved, given that in previous studies phosphorylation specifically of the second (S/T) in the motif and not dual phosphorylation was shown to be crucial for PBD binding (Elia et al., 2003, Science; Elia et al., 2003, Cell; Asano et al., 2005; Lowery et al. 2007, EMBO J; Crasta et al., 2008, Nat Cell Biol).
We have however not obtained any evidence for a priming mechanism involving SS/ST (Fig. 1B, S1C-D see also Reviewer Fig. 1). We do not observe that Cdc5 phosphorylation of Mms4 in vitro is stimulated by prior phosphorylation of Mms4 by either CDK, DDK or CDK+DDK (Fig. 1B, S1C-D). We also do not observe

any significant binding of Cdc5 to either of the two SS containing Mms4 peptides, also not in the phosphorylated state and in contrast to a known PBD-binding sequence from Spc72 (Reviewer Figure 1B). Finally, we also do not observe any two-hybrid interaction of Cdc5 and Mms4, which we would have expected, if Cdc5 robustly bound to Mms4 via its PBD.

Overall, we mention in the discussion the possibility of a Polo-box-dependent priming mechanism, but currently we lack evidence for such a mechanism. In contrast, we have substantial evidence in the manuscript that Rtt107 is involved in a mechanism with high resemblance to kinase priming as discussed below.

(2) *"Figure 3a and figure S3a; Figure 7a and Figure S7a: Why are there two sets of SILAC data?"*

We included data from the label-switch replicates in the initial submission of the manuscript. As we showed individual peptide evidences, we had to provide separate graphs, but we realized that this presentation is confusing and have therefore taken those figures out.

In the revised version of the manuscript we have now additionally included phospho-peptide data sets (Fig. 3C,D and S7b) using Mus81-Mms4 expressed from a strong promoter, which allowed us to measure Mms4 phosphorylation with much improved coverage in particular for higher order phosphorylated peptides. Furthermore, this data confirmed the trends observed with endogenous Mms4.

(3) *"Figure 4b: MMS4A-8A mutant does not interact with Cdc5 at all. This suggests that (S/T)(S/T) phosphorylation is required for Cdc5 recruitment. Reduced interaction of Cdc7 with the same mutant is surprising. Is Rtt107 bound to MMS4-8A as efficiently as the wild-type Mms4? Above hypothesis predicts that MMS4-8A may interact with Rtt107 less stably."*

Many thanks for emphasizing this important point. We have now also included a SILAC-based analysis of Mms4-8A interactors and find that Rtt107, Cdc7, Dbf4 and Cdc5 association with Mus81-Mms4 is strongly decreased in the 8A mutant (Fig. EV3). This provides first of all further evidence that Cdc5, DDK and Rtt107 form a module that binds to Mus81-Mms4 in unison. It is also consistent with the fact that we lose Rtt107 binding whenever we inhibit any of the kinases (Fig. 2E, S2A). Thus, we propose that Mms4 phosphorylation occurs via a two-step mechanism. The first requires Mms4 phosphorylation by CDK, Cdc5 and DDK thus, the second additionally involves Rtt107 and a stable tethering of Rtt107, DDK and Cdc5 to Mms4. Interestingly, initial phosphorylation of Mms4 by kinases promotes the second step via Rtt107. We therefore agree with the reviewer that our data suggest a priming step and we think this priming step promotes Rtt107 binding. We have now clarified this point in the discussion and model.

(4) *"Figure 4a and 4c and Figure 7b: The level of Mms4 has been checked. However, authors also need to examine whether association with Mus81 is affected or not."*

We are sorry for the misunderstanding, but we actually have checked the level of Mus81 in the IPs used for the in vitro cleavage experiments (Fig. S4A, EV2E, S4C-F, S5C, S7C, S7D). We also agree with the reviewer that a disruption of the Mus81-Mms4 heterodimer under specific mutant conditions would have extreme consequences on Mus81 activity. We can, however, say with confidence that the Mus81-Mms4 association is not affected by any mutant condition of this study. This can clearly be seen in the mass spec analysis of Mms4 interactors in Fig. 2E (*cdc7Δ*, controls for Fig. 4A), Fig. S6A (*rtt107Δ,* controls for Fig. 7B) and the new Fig. EV3 (*mms4-8A*, controls for Fig. 4C).

(5) *„The activity of MMS4-8A is weaker than that of the wild type but there is still significant activity in the mutant. This raises the question that Cdc7-mediated phosphorylation really regulates the enzymatic activity. Two approaches can be taken to answer this. Pretreat the purified Mms4 proteins with combinations of kinases and examine the effect on its activity. Alternatively, create the DE substitution of the putative phosphorylation sites (phosphomimic mutants) and examine the enzymatic activity.?"*

We agree with the reviewer that the *mms4-8A* mutant still shows a moderate level of Mus81 nuclease activation in mitosis (see Fig. 4C, EV2E; in particular compare with background from untagged control strain). However, we disagree with the reviewer that this would suggest that DDK would not be involved in the regulation of Mus81-Mms4 catalytic activity.

In particular, Mms4 contains many putative DDK phosphorylation sites, and not all might have been affected in the mms4-8A mutant. Consistent with this idea is our new analysis of an *mms4-12A* mutant, in which additional (S/T)(S/T) motifs were mutated. This allele showed stronger hypersensitivity towards MMS in the sgs1Δ background than *mms4-8A* (Fig. EV2D), a lower hyperphosphorylation (Fig. EV2C) and a lower, albeit not significantly, resolution activity (Fig. EV2E).

Unfortunately, phosphomimetic mutation of multiple phosphosites within Mms4 to Asp (including at several (S/T)(S/T) motifs) destabilized Mms4 (J. Matos, unpublished data) and therefore a DDK-phosphomimetic version of Mms4 is currently not achievable. On the other hand, we agree with the reviewer that an in vitro reconstitution of the phosphorylation cascade regulating Mus81-Mms4 is a worthwhile goal. We will pursue this goal in the future, but we feel that such an approach is outside of the scope of the current story.

(6) *„Does Cdc5 play any role in the enzymatic activity of Mus81-Mms4? What is the enzymatic activity of Mms4 in cdc5as mutant?"*

Yes, Cdc5 is strictly required for the activation of Mus81-Mms4 resolution activity in mitosis (see Matos et al., 2013, Cell Rep.). This data is thus completely in line with our proposed model that CDK, DDK and Cdc5 are all required to activate Mus81-Mms4 in mitosis.

(7) *"In rtt107Δ cells, the enzymatic activity is reduced, but not to the extent of cdc7Δ. Is it due to the absence of co-immunoprecipitated Rtt107? Is Rtt107 present in the immunoprecipatated fraction used for the assays? Does the presence of Rtt107 affect the enzymatic activity of Mms4? If that is the case, interpretation of these biochemical data becomes complicated.."*

Many thanks for raising this point. We have now included new experiments in order to exclude that Rtt107 which may be in the Mus81-Mms4 preparation would have an effect on resolution activity. Rtt107 is indeed present in low amounts in the Mus81 fraction used for the activity tests, however the presence of Rtt107 per se seems not to affect the enzymatic activity of Mus81. This we conclude from the experiments shown in Figure S4B-C: Using high salt (350 mM NaCl) we were able to effectively strip off Rtt107 from Mus81-Mms4 (Fig. S4B), but this did not result in a notable change in Mus81 activity (Fig. S4C). Furthermore, we now show in Figure S7D that in the *cdc7Δ bob1-1* mutant background the *rtt107Δ* mutation does not lead to a reduction in Mus81 activity. These data are therefore entirely consistent with our model that Rtt107 influences Mus81 activity indirectly by facilitating Mms4 hyperphosphorylation.

(8) *"JM resolution in vivo: It will provide stronger evidence if the authors can present data that show the JM structures cannot be resolved in cdc7Δ, mms4-8A, rtt107Δ mutants in vivo (using 2D gel)."*

While we appreciate that the 2D gel technique has given important insights into JM resolution in vivo and could as such provide further support for our model, we have decided not to conduct the suggested experiments for two reasons. First and most importantly, the 2D gel technique has two critical disadvantages – (1) it requires efficient origin firing and a relatively normal passage through S phase and (2) it is quite difficult to quantify. Of the mutants that were suggested by the reviewer *cdc7Δ* would have been very difficult to study because of its pronounced S phase defects. *mms4-8A* and *rtt107Δ* showed only partial defects in Mus81 activation and phenotypes might thus have been difficult to demonstrate/quantify by 2D gels. Second, such 2D gel experiments are rather time-consuming. Particularly, as we would have had to create a new assay system with intact DDK in S-phase and inactivation thereafter, such experiments were simply not feasible in the revision period, but will rather have to be included in follow-up work.

However, even without 2D gel data, we believe that based on the increased MMS sensitivity of *mms4-8A* and *rtt107Δ* in the *sgs1Δ* background and on the decreased CO rates in *mms4-8A* and *rtt107Δ* strains we

can conclude that Mus81-dependent JM resolution *in vivo* is partially deficient in these mutants conditions.

(Minor1) *"Page 3, subtitle: the word 'target' has extra 'e'."*

The mistake has been corrected.

(Minor2) *"Figure 3b: I apologize if I am mistaken, but is the left-right switched on this chart? I thought Cdc7 target phosphorylation decrease in the cdc7Δ cells. That means the red dots should be on the right side?"*

No, the graph is correct. However, in our phosphorylation site analysis of endogenous Mms4 we could only robustly detect singly phosphorylated (S/T)(S/T) sites, most likely a precursor for DDK phosphorylation (Fig. 3B,D). These precursors actually accumulate in the absence of DDK, since the downstream activities such as DDK were inactive.
As we could not reproducibly measure higher order (2 or more) phosphorylated peptides from endogenous Mms4, we developed an additional system using high-copy Mus81-Mms4 (Fig. 3C-D). Importantly, our new data with this system allowed us to measure singly and higher-order phosphorylated (S/T)(S/T) peptides and confirmed our previous interpretation of (S/T)(S/T) phosphorylation by DDK, as the doubly phosphorylated (S/T)(S/T) sites are less abundant in the absence of DDK, while the singly phosphorylated (S/T)(S/T) precursors are more abundant in the absence of DDK (Fig. 3C).

(Minor3) *"Figure 4f and 7c: it will be easier to understand the figures if the label is written in CO/NCO instead of the word red, red/white and white."*

We apologize for the misunderstanding. Actually, all bars in these graphs represent CO rates, the NCO rates would simply be the difference to 100%. Red, red/white and white on the other hand simply represent different classes of recombination events, dependent on the length of the conversion tract. We have now mentioned 'classes' in the figures and give a clear definition in the legend/methods.

(Minor4) *"Page 7, figure S3c was mentioned but should be S3b. "*

The mistake has been corrected.

(Minor5) *"Figure 5b, 5d: Dots on lane 3 and 6 are not necessary?"*

We have now clarified the labelling.

(Minor6) *"Figure 6a: Does Cdc5 interact with Rtt107? Why is there no interaction between Dbf4 and Rtt107?"*

Neither Dbf4, nor Cdc5 interact with Rtt107 in two-hybrid assays. For this paper and our previous work (Gritenaite et al., Genes Dev, 2014), we have done extensive interaction studies using two-hybrid, but only found few interactions (Cdc5-Dbf4; Cdc7-Rtt107; Rtt107-Slx4; Dpb11-Slx4; Dpb11-Mms4). We have not observed any evidence for bridging interactions by the endogenous complex partners, probably because the expression level of the endogenous proteins (<1000 molecules per cell) is too low to support a (bridging) two-hybrid interaction. Thus, we interpret (together with the finding that Cdc7 and Dbf4 are both found in the purifications of the complex) the finding that Cdc7, but not Dbf4 binds to Rtt107 in two-hybrid, as Cdc7 being the direct interaction partner of Rtt107.

(Minor7) *"Figure 6c: Are Cdc7 and Dbf4 present in the RTT107 IP?*

Yes, they are. In the revised version of Fig. 6C we now provide additional western blots that show that Cdc7, Dbf4 as well as Cdc5 specifically bind to Rtt107 and importantly that these interactions (in contrast to Dpb11 and Slx4) are not lost in the *slx4Δ* mutant.

(Minor8) *"Figure S2d: The shift is not very clear. I recommend the use of phostag gel to show the difference more clearly."*

While we think that it will be very interesting to see whether phosphorylation by the DDK-Cdc5 complex is a widespread phenomenon that also affects Cdc5 substrates other than Mms4, our current work does not answer this question with certainty. We agree that the data on Scc1 are not entirely clear, and even using phostag gels did not change that, but most likely Scc1 phosphorylation is slightly affected by the *cdc7Δ* mutant. Additionally, we therefore also tested another Cdc5 phosphorylation substrate (Ulp2), where the mitotic, Cdc5-dependent phosphorylation shift was clearer, but again partially dependent on DDK (Fig. S2E). Further studies will therefore be needed to clarify the impact of DDK and mitotic Cdc5 substrates in general.

(Minor9) *"Figure S5b: There are three bands for Slx4 in the input, while only one band is co-immunoprecipitated. Is there any explanation for this?" "*

We apologize for not having marked the western more clearly. Two of those are cross-reactive bands unrelated to Slx4. They have now been marked in the Figure.

Reviewer 2

We thank this reviewer for his/her enthusiasm towards our work and that he/she finds it "really exciting" and important. Moreover, we found all points raised were very thoughtful and constructive. By addressing them in full – many by new experiments, we are convinced that we were able to improve our paper significantly.

(1) *"Fig1C: Why does peptide 2 which should be di-phosphorylated run faster than monophosphorylated peptide 1? The migration profiles are more coherent for peptides 4 and 5, but what happens with peptide 6, which should run at the same position as the monophosphorylated peptide 5 once it is further phosphorylated by DDK?"*

The running behaviour of short 15mer peptides in gels (in this case a 4-12% Bis-Tris gel) is highly charge dependent, therefore we usually observe phosphorylation shifts, but both down-shifts and up-shifts are regularly observed. Importantly, the trend is consistent for both series of peptides, 1-3 shift down with increasing phosphorylation, 4-6 shift up with increasing phosphorylation. We now included a Coomassie gel of the peptides to clarify their running behaviour (Fig. S1B).
In case of peptides 4-6 we observed a low level of phosphorylation at a non-SS site, possibly the S124. In case of peptide 6 the very small signal in the autoradiograph originates therefore from a triple-phosphorylated peptide (1 hot, 2 cold), which therefore runs higher than the double-phosphorylated peptide (1 hot, 1 cold) in lane 5.

(2) *"Fig2B: Why is Mms4 still heavily phosphorylated when Cdc28 is inhibited? Shouldn't Mms4 no longer be phosphorylated in absence of Cdc28 activity and association with Cdc5 and DDK?"*

We agree with the referee and apologize for the confusion our initial experiment caused. It was done under conditions where CDK was only partly inhibited, as we used only a very low concentration (500 nM) of the inhibitor (1NM-PP1). To visualize the dose-dependent inhibition of the Mms4 phosphorylation shift, we have now included in our response a titration of 1NM-PP1 up to a concentration of 10 μM (Fig. S2B). These conditions (10 μM) have been shown to lead to effective, but specific downregulation of CDK phosphorylation in several studies (e.g. Holt et al., Science, 2009; Enserink et al., J Cell Biol, 2009) and importantly lead to a complete abrogation of the Mms4 phosphorylation shift (Fig. 2B, S2C, very similar effects are seen in Matos et al., 2013, Cell Reports), consistent with Mms4 hyperphosphorylation strictly requiring CDK. Therefore, we have also replaced the IP in Fig. 2B with a new experiment using 5 μM of 1NM-PP1, which effectively abolished Mms4 phosphorylation.

(3) *“Fig2C: Why when the Cdc5 kinase activity is completely inhibited, Mms4 is much less phosphorylated than in Fig2c even though there are detectable levels of Cdc5 and Cdc7 that associate with Mms4?”*

We have now included an experiment (Fig. S2C), where we compared the Mms4 phosphorylation shift under different mutant conditions and confirmed that inhibition of either CDK or Cdc5 largely abolished the Mms4 phosphorylation shift. Under the conditions used there is perhaps a slightly stronger effect by CDK inhibition than by Cdc5 inhibition, but this effect appears minimal. This observation is also consistent with previous work (Matos et al, 2013). Moreover, Figure 2C and Figure S2A show that Cdc5 inhibition has a very strong effect on Cdc5 and DDK binding to Mus81-Mms4 (>15 fold reduction in Fig. S2A). It cannot be excluded that residual binding of Cdc5 and DDK is responsible for a residual phosphorylation of Mms4, but this effects appear very small.

(4) *“Fig 2D: Deleting Cdc7 abolishes the Cdc5-Mms4 interaction but Mms4 still seems to phosphorylated?”*

We thank the reviewer for bringing up this point. We have made the apparent discrepant observation that certain mutants (*cdc7Δ*, but in particular *rtt107Δ*) strongly affected the association of Cdc5 and DDK, but still showed a residual phosphorylation shift of Mms4 (Fig. S2C, 2D, 6A). Conversely, we also saw that the association of Rtt107, Cdc5 and DDK, itself is dependent on phosphorylation by DDK and Cdc5.
We think that these data are best explained by a two-step model of Mms4 phosphorylation, which we now more specifically describe in the discussion of the paper. In a first step, CDK, Cdc5 and DDK phosphorylate Mms4, without being stably bound in a complex. In a second step, this phosphorylation allows Rtt107 to stably tether DDK and Cdc5 to Mus81-Mms4, which then leads to full phosphorylation of Mms4. Notably, such a mechanism has features that could be involved in generating a switch-like transition.

(5) *“Fig S2E: The authors claim that "forced expression of Cdc5 in S-phase leads to the premature occurrence of Mms4 hyperphosphorylation in S-phase".*
Although Cdc5 may indeed be the rate-limiting factor for hyperphosphorylation of Mms4, the data provided in Fig S2E don't strongly support this. Indeed in condition #6 when Cdc5 is over-expressed in S-phase cells to levels comparable to those in G2/M (conditions #4 and 7), there is only a small level of phosphorylation of Mms4 compared to conditions 4 and 7. Furthermore, FACS profile show that in condition 6 a fraction of cells are already in G2/M, which may account for the Mms4 phosphorylation.”

We appreciate the criticism and agree that in the old experiment we could not exclude with absolute certainty that a small proportion of cells had reached mitosis and would therefore disturb our measurements. We therefore switched to a different system using an inducible GFP-tagged version of

Cdc5. In Cdc5 westerns we can therefore distinguish between the accumulation of the induced $Cdc5^{GFP}$ and endogenous Cdc5. Additionally, we also use cells arrested in S-phase with hydroxyurea (HU) to stop cells from progressing into mitosis. In Figure EV1A we show that upon overexpression of $Cdc5^{GFP}$ in HU-arrested cells Mms4 becomes hyperphosphorylated. Even though there is a low level of endogenous Cdc5 present in HU-arrested cells the Mms4 phospho-shift clearly correlates with the Cdc5 levels, allowing us to conclude that indeed accumulation of Cdc5 is the limiting factor for Mms4 hyperphosphorylation.
Moreover, we use this system and compare cells that – in the presence of $Cdc5^{GFP}$ - are progressing through a "normal" or "disturbed" (HU) S phase (Fig EV1B). While replication profiles were expectedly different under both conditions, we can exclude that cells in the "normal S phase" sample had leaked into mitosis, since we did not measure any accumulation of endogenous Cdc5. Interestingly, this experiment shows that in the presence of DNA damage Mms4 hyperphosphorylation is reduced. This suggests that DNA damage signals may negatively influence Mus81 activation, perhaps due to inhibition of DDK, Cdc5 or the interaction of the two.

(6) *"The data presented in Fig 6D show that the Mms4-S201A mutant, while deficient in binding to Dpb11, is perfectly proficient in interacting with Slx4. Furthermore, Fig S6 shows that loss of Rtt107 strongly impairs binding of Slx4, but not of Dpb11, to Mms4. These results suggest that there is no relationship between Dpb11 and Slx4 in binding to Mms4. Rather than the previously proposed role for Dpb11 in bridging Slx4 and Mms4 in mitosis (Gritenaite 2014), the current study suggests that it is Rtt107 that allows the mitotic interaction of Slx4 and Mms4. Could the authors discuss this further in light of their previously published study?"*

Indeed, one of the interesting implications of our new paper is that there are two "routes" connecting Mus81-Mms4 and Slx4, one via Dpb11 (as described in our previous work, Gritenaite et al. 2014), the other via Rtt107. Currently, it cannot be said, which of them is more important and seemingly either of them is sufficient. As rightly pointed out by the reviewer in case of the *mms4-S201A* mutant, we lose binding of Dpb11 to Mus81-Mms4, but not Slx4 and Rtt107 (Fig. 6D). In case of e.g. the *mms4-8A* mutant, we lose binding of Rtt107 to Mus81-Mms4, but not of Slx4 and Dpb11 (Fig. EV3). In contrast, the experiment in Figure S6A is not informative in this regard, because we and others have observed that the *rtt107Δ* deletion mutant also abolishes the interaction between Slx4 and Dpb11 (Ohouo et al, Mol Cell 2010, our unpublished data), likely because of a defect in Slx4 phosphorylation. Therefore both "routes" to Slx4 binding are abolished in the *rtt107Δ* deletion mutant, consistent with loss of Slx4 binding. We have now specifically discussed this point in the paper.

(7) *"The authors state that DDK is not required for binding of Mms4 to Slx4 and Dpb11. However, in Fig 2E there seems to be some decrease (although modest) in the amounts of Slx4 and Dpb11 that co-purify with Mms4 in Cdc7Δ cells. Could the authors please clarify this point?"*

We thank the reviewer for pointing this out and have corrected the mistake. Indeed interaction of Mus81-Mms4 with Slx4 and Dpb11 is modestly reduced in the absence of DDK.

(8) *"Fig5: The authors claim that there is no effect of the S201A mutation on hyperphosphorylation of Mms4. This seems to be indeed the case in Fig 5B and 5D. However, phosphorylation of Mms4 S201A is reduced in Fig 5E. Moreover, regarding the HJ resolution assay with S201A, it is much more efficient in Fig 5E than in Fig 4 and Fig 7. To unambiguously claim that the S201A mutation has no effect on Mus81-Mms4 activity, this would need an in vitro nuclease assay with kinetics (and efficiency) similar to Fig 4 and Fig 7."*

The reviewer raises two important points here: (i) indeed, we observe a very small change in the electrophoretic mobility of the *S201A* mutant. As it is very small, this shift is not obvious in all gels due to differences in gel conditions used (acrylamide composition and buffer system). However, it is also obvious from our data that the hyperphosphorylation of Mms4, characteristic of mitosis, is largely intact in the S201A mutant.
(ii) as suggested by the reviewer, we have now performed a kinetic analysis of nHJ cleavage by the S201A mutant complex and compared it to WT Mus81-Mms4 (Fig. S5C, replacement of old Fig. 5E). The new data indicate that the *S201A* mutation may slightly impair Mus81 activity. However, compared to all other mutant conditions in our study, the *S201A* mutant shows only a very weak phenotype and despite repeating this experiment several times, we could not show a significant decrease in Mus81-mediated resolution. Therefore, in the revised manuscript we state that the *S201A* mutant is activated during M-phase, but that our data has the limitation of not allowing us to claim whether the activity reaches the maximum observed for wild type Mus81-Mms4.

(9) *"Why does deletion of Rtt107 only affect phosphorylation of Mms4 mediated by Cdc5 and not phosphorylation by DDK? Similarly, why does DDK deficiency not really affect Cdc5-mediated phosphorylations? Although it is clear that there is interdependency between DDK and Cdc5 for binding to Mms4, it seems that each kinase can still phosphorylate, at least partially, Mms4."*

It was indeed a weakness of the first version of our paper that conclusions like these suggested by the reviewer here could not be made, because of limited coverage of phosphorylation sites. Therefore, we now turned to a system using high-copy expression of Mus81-Mms4, where Mms4 phospho-peptides (in particular in higher order states) could be detected more reliably. This data is now included in Figure

3C,D and Figure S7B and shows that although the Cdc5 and DDK phosphorylation profiles are distinct, there is an effect of Cdc5 inhibition on the putative DDK sites (dual phosphorylated (S/T)(S/T) sites) and there is also an effect of DDK inhibition on the putative Cdc5 sites, which in our opinion nicely fits to our current model. In this new data, we also see reduced phosphorylation on putative DDK phosphorylation sites in the *rtt107Δ* mutant, although the effect of the *rtt107Δ* is very modest in these experiments in general, likely because kinase-tethering by Rtt107 acts late in the phosphorylation cascade (or potentially also because in the presence of high-level Mus81-Mms4 there is relatively little need for kinase tethering).

(10) “*This is an overall comment on the in vitro nuclease assays. Apart from data in Fig 5, the efficiency of processing of a nHJ seems surprisingly low given the fact that this is a structure that gets very efficiently processed even by recombinant Mus81-Mms4. What are the differences between reactions in Fig 5 and those shown in Fig 4 and 7?*
Are we looking here at the fully activated Mus81-Mms4 complex? Have the authors assessed the contribution of DDK to the stimulation of Mus81-Mms4 on other structures? What is the activity like of Mus81-Mms4 from S-phase cells? Based on previously published work by Tercero and colleagues it is expected to be marginal. If so, what impact does over-expression of Cdc5 in S-phase cells have on the activity of Mus81-Mms4?”

For the revised version of the manuscript we have gone through an extensive effort and introduced several controls for the Mus81-Mms4 *in vitro* assays, which in our eyes supports the statement that we are indeed measuring the activated Mus81 complex in mitosis. The seemingly low activity in our assays is simply a consequence of the fact that we IP endogenous Mus81-Mms4 and therefore use comparatively low amounts of Mus81-Mms4 in the reaction (~5 fmol of Mus81-Mms4 to cleave ~0.5 pmol of DNA substrate). This is substantially different from conventional assays using purified protein, which use ~50 fmol of Mus81 (Ehmsen and Heyer, NAR, 2008). In fact our new Figure S4A shows that Mus81-Mms4 from G2/M arrested cells is substantially more active than recombinant purified Mus81-Mms4, if similar protein concentrations are used. We are therefore confident that we are looking at fully activated Mus81-Mms4.

The seemingly higher activity of Mus81 in our old Fig. 5E was therefore simply a consequence of using more protein in the assay and as mentioned above (6), we have replaced this experiment by another, which used the same conditions as in all other cleavage assays in the paper (Fig. S5C).

Furthermore, we would like to note that Mus81-Mms4 cleaves replication fork structures similarly as the nicked Holliday Junctions and that this cleavage also depends on DDK. This new data is now included in Figure S4E.

Finally, in previous work we already showed that S-phase Mus81 is less active than M-phase Mus81 and that the S-phase activity –as well as the biological function- can be increased by premature expression of Cdc5 (Matos et al., Cell, 2011; Matos et al., Cell Rep, 2013; Gritenaite et al., Genes Dev, 2014).

(Minor1) *"Page 3: Mus81-Mms4 is a target (not "targete")"*

We have corrected the mistake.

(Minor2) *"Page 7: (See Fig S3b) (not S3C)"*

We have corrected the mistake.

(Minor3) *"dbf4-ΔN109 .... shows a strinkingly similar MMS hypersensitivity as the phosphorylation site mutant mms4-8A"*
→ Add "in the absence of Sgs1" because single mutants do not display MMS hypersensitivity?"

We have introduced the change.

Reviewer 3

We thank this reviewer for his efforts and for his constructive criticism of our work. He/she raised several specific points, all of which we have addressed in the revision. Our impression was that his/her main criticism was directed against our in vitro nuclease assays. Here, we disagree with some of the comments the reviewer voiced in point (7): as we will outline below the specific activity of Mus81-Mms4 purified from G2/M arrested cells is actually quite high, but compared to resolution assays with recombinant, purified proteins we simply use rather low protein amounts in the assays. We introduced important control experiments in support of this conclusion and thus are confident that the initial interpretations are correct. Thus, we hope that the reviewer will agree to our conclusion and support the publication of this manuscript.

(1) *"The data in Figures 1b/1c/2 do not appear to me to be internally consistent. The authors argue due to data in Figure 1c that CDK primes phosphorylation by DDK, but the evidence in Figure 1b does not support this. In Figure 2, it is shown that phosphorylation of Mms4 is abolished in cdc7 and cdc5 deficient stains - but not in cdk1 deficient cells. Moreover, in vitro kinase assays like the one shown in Figure 1b are very hard to interpret because kinases tend to phosphorylate any protein given to them. Some measure of specificity is essential."*

Our paper contains two different assays measuring specifically the phosphorylation of (S/T)(S/T) sites on Mms4. First, we measure Mms4 (S/T)(S/T) phosphorylation in vitro using peptides (Fig. 1C), second we measure Mms4 (S/T)(S/T) phosphorylation in vivo using mass spec (in particular our new phospho proteomic analysis using high-copy Mus81-Mms4 (Fig. 3). Both experiments suggest that DDK phosphorylates the first residue in (S/T)(S/T) motifs and that it becomes stimulated when the second (S/T) is phosphorylated (Fig. 1C, Fig. 3B, D). These data would therefore be consistent with a priming mechanism.

In the in vitro assay with purified Mus81-Mms4, however, we could not observe a significant priming effect by CDK, even though we have now included a new version of the kinase assay, where we bind GST-tagged Mus81-Mms4 to beads and sequentially incubate with the different kinases (Fig. 1B, S1D). We do not have a definitive explanation for the lack of CDK priming in this assay. A possible explanation is that CDK priming is actually less important for overall Mms4 phosphorylation by DDK given that only 3 (S/T)(S/T) motifs fit to the stringent CDK phosphorylation consensus and that at the other 12 (S/T)(S/T) motifs priming may be carried out by another kinase. We do not think that this is an issue of our purified kinases having low specificity in the in vitro reaction, as we have recently shown that all kinases used here in the assay show under identical reaction conditions a remarkable degree of substrate specificity (Reusswig et al., 2016, Cell Reports).

Furthermore, we apologize for the misunderstanding regarding the initial Figure 2B, which was done under conditions where CDK was only partly inhibited. As further outlined below (6), and previously shown elsewhere (Gallo-Fernández et al, NAR, 2012, Szakal et al, EMBO J, 2013, Matos et al, Cell Reports, 2013) CDK is strictly required for Mms4 phosphorylation in vivo.

(2) *"In Figure 2a the FACs data from Suppl. Figure 2a should be included and the time points indicated on the figure. Putting a series of dots over the lanes tells the reader nothing.*

We have now moved the DNA content measurement to Fig. 2A and also included the time points after release into the labelling.

(3) *"Why is the Flag input not shown in all cases in Figure 2? It should be."*

Initially, we left out the Flag input samples, as in the IPs we always pull down proportional amounts of Flag-tagged protein. However, we have now included Flag inputs for all anti-Flag IPs in the paper (Fig. 1A, 2A-D, 2F, 4B, 5B, 6A, 6C, EV2C, S2D, S2F, S4B, S5B, S6B).

(4) *"There is a consistent issue with the authors providing very little experimental detail in the manuscript body and Figure legends. For example, were the cells in Figure 2b/c/d/e arrested with nocodazole."*

We apologize. For the revised version of the paper, we have put specific attention to that all information required for understanding the experimental setup can be found in the results chapter/ figure legends.

(5) *"Scc1 is shown as a canonical Cdc5 substrate in Suppl. Figure 2d, but the blot is unconvincing."*

While we think that it will be very interesting to see whether phosphorylation by the DDK-Cdc5 complex is a widespread phenomenon that also affects Cdc5 substrates other than Mms4, our current work does not answer this question with certainty. We agree that the slight Scc1 phosphorylation shift is difficult to interpret. Additionally, we therefore also tested another Cdc5 phosphorylation substrate (Ulp2), where the mitotic, Cdc5-dependent phosphorylation shift was clearer. In both cases it seems that a partial defect in the mitotic phosphorylation shift can be observed in the absence of DDK (Fig. S2E). Further studies will therefore be needed to clarify the impact of DDK and mitotic Cdc5 substrates in general.

(6) *"In Figure 2b, inactivation of Cdk1 has only a slight effect (if any) on Mms4 phosphorylation, but abolishes Cdc5 and Cdc7 association. This is not what is described in the text."*

We apologize for the confusion, the initial experiment in Fig. 2B was done under conditions where CDK was only partly inhibited due to very low concentrations (500 nM) of the inhibitor 1NM-PP1. To better visualize the dose-dependent inhibition of the Mms4 phosphorylation shift, we have now included (Fig. S2B) a titration of 1NM-PP1 up to a concentration of 10 μM. These conditions (10 μM) have been shown to lead to effective, but specific downregulation of CDK phosphorylation in several studies (e.g. Holt et al., Science, 2009; Enserink et al., J Cell Biol, 2009) and importantly lead to a complete abrogation of the Mms4 phosphorylation shift (Fig. 2B, S2B), consistent with Mms4 hyperphosphorylation strictly requiring CDK. Therefore, we have also replaced the IP in Fig. 2B with a new experiment using 5 μM of 1NM-PP1.

(7) "The biochemistry described in Figures 4 and 7 is very weak. There are 2 major issues. First, the incubation timers are very long, indicative of very weak nuclease activity. Second, the product bands are so faint - as to be nearly impossible to interpret. In particular, in Figure 7, I would doubt if the activity is altered much, if at all. These experiments need to be repeated to get decent levels of cleavage (> 50%). Also, it is not acceptable to plot the graphs with the vertical axis not beginning at zero. This is done presumably to try to make any tiny difference look impressive."

Here, the reviewer raises two related points of concerns. (i) that the measured nuclease activity would be low and (ii) that because of this low activity some effects of mutants may be difficult to interpret. Supported by new control experiments, we would like to contradict this criticism.

(i) the nuclease activity of Mus81-Mms4 used in our assays is not "very weak", we simply use rather low amounts of protein (~5 fmol Mus81-Mms4) in order to cleave DNA structures that are in large excess (~0.5 pmol). This is due to the fact that we are working with Mus81-Mms4 that was endogenously expressed in yeast and purified from M-phase arrested cells by IP. Previous assays in the literature have worked with much higher amounts of Mus81-Mms4 (50 pmol, Ehmsen and Heyer, NAR, 2008) leading to higher substrate turnover. However, when we carefully compare the specific activity of Mus81-Mms4 purified by IP, it is actually higher compared to what was previously determined with purified protein (Fig. S4A) consistent with cell cycle-dependent activation of the protein.

(ii) while we agree that higher substrate turnover (>50%) would make some of the effects look visually more clear cut on the gel picture, we disagree that only a repetition under conditions of higher substrate turnover would allow meaningful conclusions. We can accurately quantify the data from the gel pictures and the graphs for the key experiments in Figure 4A, 4C and 7A are in fact averages from three independent experiments, showing that the effects measured are indeed reproducible.

Lastly, we agree with the reviewer that the graphs in the first version were not done properly: axes should always start at 0. Just for clarification, we did not do this with the intention of deceiving the reader, as implied by the reviewer, but rather by mistake (it arose because of a faulty background

substraction). Axes now include the origin and we also now included a background subtraction. Although this did neither change the appearance of the graphs very much nor the result, we would like to apologize for the initial presentation of the graphs. We also agree that the effect of the *rtt107Δ* mutant on Mus81 activity is less pronounced compared to a *cdc7Δ,* consistent with a non-essential role of Rtt107 in the function of Mus81-Mms4. We apologize if this was not properly mentioned in the first version of the manuscript, it will be in the new version.

(8) *"In Figure 4, why was residue 286 not mutated? It looks like the best candidate to my eye?"*

Phosphorylation of serine 286 is highly dependent on Cdc5 (as well as DDK). Indeed, it is one of the best candidates for a Cdc5 target site on Mms4. We therefore generated an mms4-S286A mutant (single and in combination with mutants affecting other putative Cdc5 sites). However, these mutants showed only a slightly increased MMS hypersensitivity when combined with *sgs1Δ*. Moreover, this mutant also shows a partial defect in Cdc5 and DDK association. While we think that such phenotypes might be interesting to investigate further, it is also clear that we have not yet identified a "complete" Cdc5 phosphorylation-deficient version of Mms4 and that it would be premature to include this data in the manuscript (see Reviewer Figure 2).

(9) *"Figures 5b and 5d could be combined."*

Yes, we agree and have combined the figures.

2nd Editorial Decision 06 December 2016

Thank you for submitting your revised manuscript for our consideration. Two of the original referees have now evaluated it again, and I am pleased to say that both consider the study significantly improved and now in principle acceptable for publication. Nevertheless, referee 2 still has a few queries and concerns, which I would kindly ask you to respond to and address in an additional, final round of minor revision.

REFEREE REPORTS

Referee #2:

Overall the authors have done an excellent job at answering my concerns and, from what I can tell, those of the other reviewers.
The manuscript has been under quite some modifications with remarkable efforts from the authors to add additional data to answer the reviewers comments.
This study deserves to be published as it will undoubtedly be of interest to a large reader ship on a timely topic.
I still have a few questions/comments that are listed below.

1- Figure 2D and S2E: the migration profile of Mms4 in absence of Cdc7 is quite different with a marked doublet in S2E that is not seen in 2D. Why is this?

2- Figure EV1B what would happen in an slx4-delta? Based on work by the Smolka lab, Slx4 is needed to dampen the checkpoint. In absence of Slx4, would this result in a stronger checkpoint and thereby stronger inhibition of DDK, which subsequently should lead to reduced phosphorylation levels of Mms4?

3- Figure S3A: The authors suggest (page 7 top) that it is the PPase treatment of their recombinant Mus81-Mms4 samples that "kills" their nHJ processing activity? Treating the endogenous Mus81-Mms4 sample with PPase is expected to similarly reduce its activity. It would be worth having this control in this figure. The way things are explained seem confusing to me as it looks like the recombinant complexes immunoprecipitated from S. cerevisae undergo a dephosphorylation step (cf above and page 7 top and Fig S3A). I assume that this is not the case for the yeast produced recombinant samples used in the following experiments.

4- Figure S3C: My understanding is that the salt washes were done on the recombinant complex. If so, why wasn't this done also on the endogenous complex? Does washing the endogenous complex with a high salt buffer have any impact on the activity of the endogenous complex? This is an important point as results from such experiments could argue towards phosphorylation direclty mediating catalytic control of the Mus81-Mms4 complex or towards co-factors bindings to the phosphorylated complex.

5- Figure 4: There is a problem in the legend on the graph, at least on my PDF.

6- Figure 4F: The mms4-delta control should have been included so that the reader can assess to which extent the levels of CO events are reduced in the mms4-8A mutant

7- Figure 7: there are problems with the legends in the graphs of 7B and C

8- Figure 7B: same as Figure 4F above.

9- General comment. The authors show that Mus81 is also phosphorylated, yet the entire work focuses on the phosphorylation of Mms4 with, in many instances, phenotypes associated with Mms4 phosphorylation mutants that are less dramatic than anticipated. This could be due to the fact that not all phosphorylation sites are mutated, but could also be explained if phosphorylation of Mus81 is also important. I am not sure that this point was considered/discussed.

Referee #3:

Although I am still not fully convinced by the less than ideal biochemical data, I believe that the authors have made decent attempt to improve their manuscript in response to the reviews. I therefore recommend that the manuscript now be accepted.

2nd Revision - authors' response 15 December 2016

Point-by-point response (Princz et al.) – EMBOJ-2016-94831

Reviewer 2

We thank the reviewer for praising the revised version of our manuscript and very carefully for checking our manuscript again. We hope that our answers in the point-by-point response clarify his/her remaining questions, particularly a misunderstanding regarding the in vitro resolution assays.

Specific points:

(1) *"Figure 2D and S2E: the migration profile of Mms4 in absence of Cdc7 is quite different with a marked doublet in S2E that is not seen in 2D. Why is this?"*

Two different gel systems were used. For Figure 2D we used a 4-12% Bis-Tris gradient gel, while for S2E we used a 7% Tris-Acetate gel. In the 7% gels the different phosphorylated species of Mms4 are better resolved and a doublet is observed in the absence of DDK. This doublet is compacted, but visible in the 4-12% gels (Fig. 2D). We now specifically mention, in which figures the 7% gels where used in the corresponding legends as well as the materials and methods section of the paper.

(2) *"Figure EV1B what would happen in an slx4-delta? Based on work by the Smolka lab, Slx4 is needed to dampen the checkpoint. In absence of Slx4, would this result in a stronger checkpoint and thereby stronger inhibition of DDK, which subsequently should lead to reduced phosphorylation levels of Mms4?"*

It is difficult to predict what would happen in such a scenario. Indeed *slx4* mutants generally show a hyperactive checkpoint, which may lead to a stronger inhibition of DDK. It needs to be mentioned, however, that this hyperactivation is dependent on formation of the Dpb11-dependent checkpoint complex. The setup of artificial Cdc5 expression in S phase (Fig. EV1B) could thus also influence the phenomenon of "checkpoint dampening" as well, as it seems for example likely that Mms4 will interact with Dpb11 under these conditions, which in turn may influence the ability of Slx4 to

regulate the checkpoint. Given the pleiotropic effects of Cdc5 expression in S phase it thus seems somewhat doubtful that a mutant analysis under these conditions will allow strong conclusions on the interplay of checkpoint and JM resolution in WT cells.

(3) *"Figure S3A: The authors suggest (page 7 top) that it is the PPase treatment of their recombinant Mus81-Mms4 samples that "kills" their nHJ processing activity? Treating the endogenous Mus81-Mms4 sample with PPase is expected to similarly reduce its activity. It would be worth having this control in this figure. The way things are explained seem confusing to me as it looks like the recombinant complexes immunoprecipitated from S. cerevisae undergo a dephosphorylation step (cf above and page 7 top and Fig S3A). I assume that this is not the case for the yeast produced recombinant samples used in the following experiments."*

We (J. Matos & M. Blanco) have previously shown that PPase treatment abolishes Mus81 resolution activity under almost identical assay conditions (endogenously purified Mus81-Mms4, see Fig. 6C in Matos et al, 2011, Cell). Conversely, the Heyer lab has shown that recombinant Mus81-Mms4 can be activated by phosphorylation (Schwartz et al, 2012, MCB). Therefore, the difference in activity between the endogenous (phosphorylated) and the recombinant (PPase-treated) Mus81-Mms4 is easily explained by the difference in phosphorylation.
Furthermore, there is unfortunately a misunderstanding here (see below, (4)). All JM resolution experiments in the paper were performed with endogenous Mus81-Mms4. The recombinant (PPase treated) complex was just used as control in Figure S4A to demonstrate that the relative activity of the endogenous, affinity-purified complex is comparably high. We have rewritten the results chapter to clarify this point.

(4) *"Figure S3C: My understanding is that the salt washes were done on the recombinant complex. If so, why wasn't this done also on the endogenous complex? Does washing the endogenous complex with a high salt buffer have any impact on the activity of the endogenous complex? This is an important point as results from such experiments could argue towards phosphorylation direclty mediating catalytic control of the Mus81-Mms4 complex or towards co-factors bindings to the phosphorylated complex."*

Unfortunately, this is indeed a misunderstanding. The salt washes were done on the endogenous complex (Fig. S4B,C) as a control that co-purifying Rtt107 does not influence the activity. All resolution assays in the paper were done using the endogenous complex. The recombinant complex was only used once as a general control for the activity in Fig. S4A.

(5) *„ Figure 4: There is a problem in the legend on the graph, at least on my PDF."*

Problem with pdf conversion. Fixed.

© European Molecular Biology Organization

(6) *„ Figure 4F: The mms4-delta control should have been included so that the reader can assess to which extent the levels of CO events are reduced in the mms4-8A mutant.“*

Agreed, we have now included the *mms4Δ* mutant in Figure 4F. It shows a reduction in CO rates that is very similar to what previously has been described for *mus81Δ* (Ho et al. 2010, Mol Cell). Importantly, as stated in the text, the rates of CO formation in either *mms4Δ* (Fig. 4F) or *mus81Δ* (Ho et al. 2010, Mol Cell) are indeed lower than in the *mms4-8A* mutant.

(7) “*Figure 7: there are problems with the legends in the graphs of 7B and C.”*

Problem with pdf conversion. Fixed.

(8) “*Figure 7B: same as Figure 4F above.”*

See (6). We have now included the data on the *mms4Δ* mutant in Figure 4F.

(9) *“General comment. The authors show that Mus81 is also phosphorylated, yet the entire work focuses on the phosphorylation of Mms4 with, in many instances, phenotypes associated with Mms4 phosphorylation mutants that are less dramatic than anticipated. This could be due to the fact that not all phosphorylation sites are mutated, but could also be explained if phosphorylation of Mus81 is also important. I am not sure that this point was considered/discussed.”*

Agreed. We observe phosphorylation of Mus81 both in vitro as well as in vivo. Phosphorylation of Mus81 could thus play a role in the cell cycle regulation of Mus81-Mms4. We have now acknowledged this possibility in the discussion.

3rd Editorial Decision 19 December 2016

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Boris Pfander
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2016-94831

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures
1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo
http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title
http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun
http://datadryad.org
http://figshare.com
http://www.ncbi.nlm.nih.gov/gap
http://www.ebi.ac.uk/ega
http://biomodels.net/
http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Most biochemical and genetic experiments in this study are bulk experiments, measuring behaviour of at least 1x10^7 cells or 5 fmol molecules. The only exception are the genetic crossover assay, which was done with several hundred cells, which empirically showed highly similar effects over independent biological replicates in our hands and the literature.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	NA
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Antibodies and their sources are described in the Materials and Methods section. For commercially available antibodies respective order numbers are depicted.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	mass spectrometric data will be made available via EBI PRIDE ....
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	included in Data Availability: "Mass spectrometric datasets available at EBI PRIDE. Dbf4-dependent kinase (DDK) and the Rtt107 scaffold promote Mus81-Mms4 resolvase activation during mitosis (2015). PXD005356."
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA