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Structure of the *S. cerevisiae* Hrr25:Mam1 monopolin subcomplex reveals a novel kinase regulator

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Transaction Report:

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

1st Editorial Decision 08 March 2016

Thank you for submitting your manuscript on Hrr25-Mam1 monopolin subcomplex structure for our consideration. It has now been reviewed by three expert referees, whose comments are copied below for your information. As you will see, the referees appreciate the quality and potential interest of your new data for the field, but also raise concerns about the generality and scope of the results as presented now.

Having now discussed these reports and recommendations within our team, we had to conclude that the study is at present not sufficiently far-reaching for a full EMBO Journal article. We do however realize that the work may become a stronger candidate if you should be willing and able to extend it along the lines suggested by the referees. This would include decisive additional structure-guided functional/mechanistic experiments, especially using Hrr25-Mam1 interaction mutants (see referee 1 points 4-6, referee 2 point 6, and referee 3's general comments). Moreover, we feel that for EMBO Journal publication, testing some of the functional implications of the current structural and biochemical work in vivo in yeast would also be essential (see referee 3's two concrete suggestions).

I would therefore like to give you an opportunity to address these issues through a revised version of the manuscript. Since we allow only for a single round of major revision, it will however be important to comprehensively answer to all the referee and editorial points during this round. Should it be helpful, I would therefore be open to discussing an extension of the revision deadline in this case. We usually allow for three months revision, and it is our policy that competing manuscripts published elsewhere during such an official revision period would have no negative impact on our

final assessment of your revised study. Finally, should you have any comments/questions regarding the referee reports or this decision, please do not hesitate to contact us.

Thank you again for the opportunity to consider this work for The EMBO Journal, and I look forward to hearing from you in due time.

REFEREE COMMENTS

Referee #1:

Ye et al report the structure of the Hrr25 kinase, a casein kinase (CK) family member required for sister kinetochore co-orientation during meiosis I. Several structures are presented in differing ligand or activation states as well as a complex with a section of the monopolin subunit, Mam1. Some kinetic characterizations of the proteins as well as an in vitro assay against the presumed physiological substrate complete the study.

Overall, I found this an interesting and and straightforward manuscript. It seems technically sound, though I have some reservations about the interpretation of the kinetic data (below). The principle novelty lies in the details of the central insertion in the kinase and the mode of binding to Mam1. The tethered recruitment of the kinase and presumed cis-inhibition of monopolin binding within each kinetochore is a nice idea and fits with the general structural model. Nevertheless, it should be noted that the generality of this mechanism is not clear, as the monopolin complex is highly diverged and the model is still rather speculative. Whether these results are of sufficient wider interest should therefore be an editorial decision. Regardless, I have a number of specific points that should also be addressed:

1. The crystallized construct contains the N-terminal and "central" domain. It is not clear from the manuscript what the predicted structure/function of the C-terminal domain of Hrr25 is. A little more explanation here would clarify the manuscript for the non-specialist reader.

2. There is considerable discussion of the bound ions - phosphate or sulfate. Nowhere is any electron density shown for these regions of the structure. Appropriately scaled difference maps at least should be shown in the supplemental materials to support these assignments.

3. Similarly, the Hrr25 central domain is described as having a high degree of thermal motion and being partially disordered. In the absence of coordinates and structure factors, this is hard for a reader to assess. Presentation of appropriate electron density for these regions would allow the reader to assess the quality of the modelled structure.

4. I'm not entirely convinced by some of the assertions presented on the basis of the kinetic data obtained. The authors argue that the binding of Mam1 to Hrr25 increases the specificity of the kinase to its physiological substrate solely on the basis of the observation the the complex has a higher Km (i.e. lower affinity) against a non-specific substrate than the free Hrr25. This might be true, but other explanations for this behavior are also possible. A proper competition assay employing the "correct" substrate would be required to robustly justify this claim.

5. A number of mutations are described which affect binding of Mam1 to Hrr25. How do these mutant proteins behave in the kinase assay? Do the mutants affect the kinetic parameters of the complex?

6. The phosphorylation of the Mtw1 complex is shown by a gel shift assay, where a change in the mobility of Dsn1 is observed upon addition of Hrr25/Hrr25-Mam1. The phosphorylation of Dsn1 seems robust although it would have been nice if an -ATP control had been included in the assay. The claim the the Hrr25:Mam1 complex is more active seems a bit hard to justify from the gel presented. Although there is a slight apparent super-shift of Dsn1 with the complex relative to apo-Hrr25 it is not that pronounced, and the relative loading of the kinase in the two reaction conditions is not clear from the gel. Also, should there be a band for Mam1 present? While I have no problem

with the overall message, I think a more quantitative treatment would be required to really nail-down the specificity argument.

Referee #2:

Kevin Corbett is probably the only structural biologist who focuses on meiotic proteins. His previous work has been very useful and inspiring for many people studying meiosis. Similarly, the current manuscript reports findings that are important for the field of meiosis but also for other fields where Hrr25 plays roles. Solving the structure of Hrr25/Mam1 monopolin subcomplex is a major achievement. One of the important impacts of this structure is that it allows for the first time to assemble structural features of the whole monopolin complex. The authors also nicely discuss how these structural features may affect monopolin function although in vivo experiments are missing. The manuscript is suitable for the EMBO Journal after addressing the following points.

Major concerns:

1) The manuscript is focused too much on the budding yeast and it gives an impression that these results are relevant only to budding yeast and other closely related yeast species. The role of casein kinase 1 delta/epsilon in mono-orientation of sister kinetochores during meiosis I should be extended to other organisms. This does not have to be addressed experimentally but one additional paragraph should be added to the Discussion. This should include studies of Hrr25 homologs and their role in recombination and mono-orientation of sister kinetochores in fission yeast (T. Sakuno et al., Dev Cell 2015; N. Phadnis et al., PLoS Genet 2015; A. Dudas et al., Cell Cycle 2011; Y. Hirose et al., PLoS Genet 2011).

Minor concerns:

1) When monopolin is mentioned for the first time in the Introduction, K. Rabitsch et al., Dev Cell 2003 and A. Toth et al., Cell 2000 should be cited. These were the paper that discovered and named the monopolin complex.

2) The observation that there is a strong correlation between the presence of the Hrr25 central domain, Mam1, and point centromeres is exciting but it is not clear what does this mean. I would like to encourage the authors to add more speculations and ideas discussing this observation. For example, why Mam1 and Hrr25 central domain are dispensable in organisms with a more complex centromere.

3) Please explain why autophosphorylated Hrr25 is not suitable for crystallization trials, why CK1-7 inhibitor was added and what is B-factor. This will help readers who are not experts in structural biology to understand experiments.

4) There seems to be a mistake in the last sentence of the Abstract (I think that "kinetochore" should be deleted)

5) The observation that Hrr25 kinase activity is altered by Mam1 binding is not novel. This was one of the conclusions of Kevin Corbett's previous paper (Corbett and Harrison, Cell 2012). This does not make the current study less important but it should be clearly mentioned in the text.
This also means that the title of the manuscript is misleading because this study has not identified a novel regulator of Hrr25. I suggest to change the title.

6) I understand that it is not easy to purify Mtw1 complex but if the authors meanwhile improved their purifications, it would be useful to test if Mtw1 complex alters Hrr25 kinase activity.

7) Please discuss whether the structure of Mam1 is similar to other proteins.

8) Hrr25 should be labeled in the Figure 8B.

Referee #3:

Corbett and colleagues report on the structure of an interface in the monopolin complex, a crucial kinetochore complex that ensures co-orientation of sister chromatids during meiosis I in yeast. Crystal structures of the Ck1δ kinase Hrr25 from two different organisms and a structure of Hrr25 in complex with a conserved segment of Mam1 reveal the organization of the kinase domain and extensive interaction interfaces with Mam1. Further biochemical experiments suggest that Mam1 specifically enhances Hrr25 kinase activity towards the kinetochore substrate Dsn1, but not to a non-physiological substrate, suggesting that the type of interface formed is critical for Hrr25 acitivity.

The monopolin complex is a fascinating part of the meiotic kinetochore and the structural work presented by the corresponding author in previous papers has already provided important functional insights. There are a number of questions that remain unanswered regarding monopolin and Hrr25 function: What are the relevant substrates of the kinase and how does phosphorylation contribute to Monopolin function? The V-shaped structure of the core assembly is very suggestive for its role as a cross-linker, but how does the cell ensure that sisters are cross-linked and not intra-kinetochore cross-linking occurs? This is puzzling, as each kinetochore contains multiple copies of the potential monopolin receptor Dsn1 (a subunit of the Mtw1 complex) and it is not at all obvious how co-orientation can be efficiently achieved.

Major point:

The structure of the Hrr25 kinase domain and the Hrr25-Mam1 complex provide novel insights into the kinase fold and how it relates to other kinases of this type. The extensive interface formed between Hrr25 and Mam1 explains the tight binding of the kinase to the rest of the monopolin complex. The crystallographic data are well presented and the interface between Hrr25 and Mam1 is validated using mutants in pull-down assays. The experiments are very well performed and accurately described in the paper.

A major shortcoming is that the paper does not go very much beyond the structural description of the Hrr25-Mam1 interface. Especially since budding yeast proteins are employed, the reader is left a bit frustrated that none of the crystallographic insights is validated or analyzed with mutants in vivo. As the presented structures per se do not immediately provide or suggest answers to the questions formulated above, the impact of the manuscript in its current form is limited by the lack of functional experiments.
The authors entertain the possibility that Mam1 enhances specificity and catalytic activity of Hrr25 towards relevant kinetochore substrates, and preliminary experiments with the Mtw1 kinetochore complex are presented. The consequences of Dsn1 phosphorylation by Hrr25, however, remain undefined and phosphorylation of Mam1 (previously suggested to be critical for monopolin function) is not further investigated.
In summary, the authors could increase the impact of the study by analyzing structure-defined mutants in the Hrr25-Mam1 interface for meiotic defects in vivo. Alternatively, the mechanistic consequences of Dsn1 phosphorylation could be studied further. I feel that for a journal with an emphasis on mechanistic insights, such as EMBO J, this type of extensions of the structure biology experiments would be required to support publication.

1st Revision - authors' response 03 July 2016

Responses to Referee’s comments

We appreciate the referees’ supportive assessment of our work, and their comments on aspects that could be improved. Below we respond to each referee’s points in detail, with notes as to where changes to the manuscript have been made in each case. The attached revised manuscript has these (and all other) changes highlighted in red text.

Referee #1:

... Nevertheless, it should be noted that the generality of this mechanism is not clear, as the monopolin complex is highly diverged and the model is still rather speculative.

It is true that the monopolin complex is not involved in meiotic sister kinetochore co-orientation outside a small group of fungi. The phenomenon of sister kinetochore co-orientation is, however, conserved throughout eukaryotes, and the molecular mechanism of co-orientation remains largely mysterious in all systems. Thus, work on the monopolin complex can provide a useful paradigm for understanding sister co-orientation in one relatively "simple" context.

1. The crystallized construct contains the N-terminal and "central" domain [of Hrr25]. It is not clear from the manuscript what the predicted structure/function of the C-terminal domain of Hrr25 is. A little more explanation here would clarify the manuscript for the non-specialist reader.

The C-terminal ~100 residues of Hrr25 is highly enriched in proline and glutamine (hence it is referred to as the "P/Q-rich" region in Figure 1A), and is predicted to be mostly disordered in solution. We have added further explanatory text on page 4 making this point more clearly. However, spurred by this question, we cloned and attempted to purify full-length *S. cerevisiae* Hrr25, and its complex with Mam1, for biochemical analysis. We were not able to purify sufficient amounts of full-length Hrr25 alone for analysis, but we were able to purify its complex with Mam1$^{87\text{-}191}$. This preparation is less pure than the truncated constructs as the C-terminal region is highly sensitive to proteolysis, supporting the idea that it is largely disordered. ATPase assays using this complex in the presence of casein show that the C-terminal domain does not significantly alter the *in vitro* kinase activity of Hrr25:Mam1. These results are now shown in Figure 7, and described on page 8 of the manuscript.

2. There is considerable discussion of the bound ions - phosphate or sulfate. Nowhere is any electron density shown for these regions of the structure. Appropriately scaled difference maps at least should be shown in the supplemental materials to support these assignments.

We have added an additional panel to Supplemental Figure 3 (panel A) showing electron density from a simulated-annealing omit map from our 2.0 Å-resolution structure of *C. glabrata* Hrr25 bound to ADP, showing the sulfate ion bound to site #1. The electron density for the sulfate ion bound to site #2 is of similar quality and I/σ value. In both cases, the assignment of this density to a sulfate ion was made on the combined basis of: 1) what ions were most prevalent in the crystallization solution, 2) the shape of the density at low I/σ, and 3) the high I/σ of the central atom (in this case, 8.5 σ in a $2F_o$-F_c SA-omit map).

3. Similarly, the Hrr25 central domain is described as having a high degree of thermal motion and being partially disordered. In the absence of coordinates and structure factors, this is hard for a reader to assess. Presentation of appropriate electron density for these regions would allow the reader to assess the quality of the modelled structure.

We have added an additional panel to Supplemental Figure 3 (panel B) showing the electron density (or rather, lack thereof) for the loop between residues 302 and 339 in our 2.0 Å-resolution structure of *C. glabrata* Hrr25 bound to ADP. This map shows clear density for the alpha-helices on either side of the loop, but no interpretable density for the loop itself, which includes α-helix 2. Also, we would note that the four structures of isolated Hrr25 described in this manuscript – including PDB and structure factor files – are publicly available from the Protein Data Bank. The PDB code for the structure shown in both Supplemental Figure 3A and B is 4XH0 (see **Supplemental Table 1** for all PDB codes). The two structures of Hrr25:Mam1 have been assigned PDB codes and are scheduled to be publicly released on July 31, 2016 (or upon manuscript acceptance). Original diffraction data for all structures (hosted by the SBGrid Data Bank) will also be publicly released at that time.

4. I'm not entirely convinced by some of the assertions presented on the basis of the kinetic data obtained. The authors argue that the binding of Mam1 to Hrr25 increases the specificity of the kinase to its physiological substrate solely on the basis of the observation the the complex has a higher Km (i.e. lower affinity) against a non-specific substrate than the free Hrr25. This might be

true, but other explanations for this behavior are also possible. A proper competition assay employing the "correct" substrate would be required to robustly justify this claim.

We share the reviewer's skepticism on this point, and to more directly address the question of specificity, we sought to identify a more specific substrate. Because we have been unable to purify large enough quantities of the intact Mtw1 complex for use in stimulation assays, we instead purified the Csm1-binding region of Dsn1 (residues 51-120) fused to *E. coli* maltose-binding protein. Unfortunately, this construct also failed to stimulate Hrr25 or the Hrr25:Mam1 complex in our assays. A construct containing the entire N-terminal region of Dsn1, residues 1-234, was too proteolytically-sensitive to purify in sufficient quantities to test in these assays. We also failed to observe stimulation by a peptide covering Mam1 Ser214, previously identified as strongly phosphorylated by Hrr25. Because we have not observed that Mam1 mediates an increase in affinity for any substrate, only a decrease in affinity for casein, we have softened this point in the manuscript (page 10, second paragraph).

5. A number of mutations are described which affect binding of Mam1 to Hrr25. How do these mutant proteins behave in the kinase assay? Do the mutants affect the kinetic parameters of the complex?

This experiment was not attempted prior to the original submission because mutations that disrupt Mam1-Hrr25 binding would not be expected to support co-expression and purification of the mutant complexes from *E. coli*. To test this, we have now cloned several mutations of Mam1 into our co-expression vector and tested whether purification of these mutant complexes is possible. The results (now shown in Figure S5B) indicate that these Mam1 mutations do not co-purify with Hrr25 when co-expressed (also noticeable is a distinctly lower yield of Hrr25 in these cases, supporting Hrr25 stabilization by Mam1). Thus, it is essentially impossible to perform ATPase assays with these mutant complexes. The exception is Mam1 R131A, whose mutation does not affect *in vitro* activity, as shown in the original manuscript.

6. The phosphorylation of the Mtw1 complex is shown by a gel shift assay, where a change in the mobility of Dsn1 is observed upon addition of Hrr25/Hrr25-Mam1. The phosphorylation of Dsn1 seems robust although it would have been nice if an -ATP control had been included in the assay. The claim the the Hrr25:Mam1 complex is more active seems a bit hard to justify from the gel presented. Although there is a slight apparent super-shift of Dsn1 with the complex relative to apo-Hrr25 it is not that pronounced, and the relative loading of the kinase in the two reaction conditions is not clear from the gel. Also, should there be a band for Mam1 present? While I have no problem with the overall message, I think a more quantitative treatment would be required to really nail-down the specificity argument.

The referee is correct that it's difficult to say with certainty that Hrr25:Mam1 is more active than Hrr25 based on the results shown. Thus, we have altered the text on page 8 to note that, given the data we have, the complex appears roughly as active as Hrr25 alone. We regret the omission of the (-) ATP control; this was done in earlier experiments, along with experiments omitting $MgCl_2$ and adding calf intestinal phosphatase; these all showed the expected results but are not shown due to poor resolution on Phos-tag gels. Nonetheless, the suppression of the Dsn1 band-shift by CK1-7, a specific CK1 inhibitor, is compelling evidence of specificity. With regard to a gel band for Mam1, the Phos-tag gels we ran to efficiently visualize Dsn1 phosphorylation resulted in the Mam1 band (~12 kDa) running off the gel.

Referee #2:

The manuscript is focused too much on the budding yeast and it gives an impression that these results are relevant only to budding yeast and other closely related yeast species. The role of casein kinase 1 delta/epsilon in mono-orientation of sister kinetochores during meiosis I should be extended to other organisms. This does not have to be addressed experimentally but one additional paragraph should be added to the Discussion. This should include studies of Hrr25 homologs and their role in recombination and mono-orientation of sister kinetochores in fission yeast (T. Sakuno et al., Dev Cell 2015; N. Phadnis et al., PLoS Genet 2015; A. Dudas et al., Cell Cycle 2011; Y. Hirose et al., PLoS Genet 2011).

The impression that our results are mainly relevant to budding yeast is no accident; indeed, the fact that both the Hrr25 central domain and Mam1 are found only in budding yeast speak strongly to exactly this point. We have altered the introduction to clarify the species distribution and roles of the various monopolin complex subunits (Page 3), hopefully making it more clear that our results apply mainly to budding yeast. We have also clarified the different roles of the conserved monopolin subunits Csm1 and Lrs4 in budding versus fission yeast.
Nonetheless, as the reviewer notes it is important to point out the other meiotic roles of CK1δ kinases in both budding and fission yeast. We have therefore added a new section to the introduction noting these functions, with the references noted above (Page 4).

1) When monopolin is mentioned for the first time in the Introduction, K. Rabitsch et al., Dev Cell 2003 and A. Toth et al., Cell 2000 should be cited. These were the paper that discovered and named the monopolin complex.

We have added these references (plus Petronczki et al. *Cell* 2006) at the first mention of the monopolin complex.

2) The observation that there is a strong correlation between the presence of the Hrr25 central domain, Mam1, and point centromeres is exciting but it is not clear what does this mean. I would like to encourage the authors to add more speculations and ideas discussing this observation. For example, why Mam1 and Hrr25 central domain are dispensable in organisms with a more complex centromere.

While all organisms must co-orient sister kinetochores in meiosis I, only in budding yeast is this mediated by the monopolin complex. We speculate that the re-purposing of the monopolin complex to mediate meiosis I sister co-orientation occurred coincident with the evolution of the point centromere, which presumably also disrupted whatever prior mechanism was in place to ensure accurate kinetochore-microtubule attachments in meiosis I. As we have no direct evidence for these points, however, we prefer not to speculate further than is already included in the manuscript.

3) Please explain why autophosphorylated Hrr25 is not suitable for crystallization trials, why CK1-7 inhibitor was added and what is B-factor. This will help readers who are not experts in structural biology to understand experiments.

An important consideration when judging suitability for crystallization is homogeneity of the sample, both chemical and conformational. We reasoned that because the autophosphorylation of *E. coli*-expressed Hrr25 is heterogeneous, it would likely inhibit crystallization. This was mentioned in the original text (page 4). For similar reasons, CK1-7 binding likely reduced the motion of the kinase domain N-lobe, thus contributing to conformational homogeneity. We now mention this more directly on page 4. Also, we have included a rough working definition of the crystallographic B-factor on page 6.

4) There seems to be a mistake in the last sentence of the Abstract (I think that "kinetochore" should be deleted)

We thank the reviewer for catching this error, and have corrected it.

5) The observation that Hrr25 kinase activity is altered by Mam1 binding is not novel. This was one of the conclusions of Kevin Corbett's previous paper (Corbett and Harrison, Cell 2012). This does not make the current study less important but it should be clearly mentioned in the text. This also means that the title of the manuscript is misleading because this study has not identified a novel regulator of Hrr25. I suggest to change the title.

The reviewer is correct that we previously observed an effect on Hrr25 activity by Mam1. We now more explicitly mention this on page 8 of the manuscript. With regard to the title of the manuscript, the word “novel” in the title refers mainly to the structural novelty of Mam1, which to our knowledge is structurally unlike any kinase regulator characterized to date. In addition, the probable direct regulation of Hrr25 by Mam1 Arg131 is a novel finding. We therefore feel that the title is appropriate, but are willing to defer to the editor’s view.

6) I understand that it is not easy to purify Mtw1 complex but if the authors meanwhile improved their purifications, it would be useful to test if Mtw1 complex alters Hrr25 kinase activity.

As noted above, we have unfortunately been unable to purify the Mtw1 complex in the amounts required to test whether it stimulates Hrr25 directly in our ATPase assays. As mentioned above, we did test Hrr25 and Hrr25:Mam1 activity in vitro in the presence of the isolated N-terminal region of Dsn1, but failed to observe any stimulation of ATPase activity. We agree with the reviewer that this is an important avenue for future study.

7) Please discuss whether the structure of Mam1 is similar to other proteins.

As with the Hrr25 central domain, the structure of Mam1 is novel, with no similar proteins identified with structural similarity searches such as the DALI server. We now mention this point on page 6 of the revised manuscript.

8) Hrr25 should be labeled in the Figure 8B.

We have added a label to each copy of Hrr25 in this figure.

Referee #3:

... A major shortcoming is that the paper does not go very much beyond the structural description of the Hrr25-Mam1 interface. Especially since budding yeast proteins are employed, the reader is left a bit frustrated that none of the crystallographic insights [are] validated or analyzed with mutants in vivo. As the presented structures per se do not immediately provide or suggest answers to the questions formulated above, the impact of the manuscript in its current form is limited by the lack of functional experiments.
The authors entertain the possibility that Mam1 enhances specificity and catalytic activity of Hrr25 towards relevant kinetochore substrates, and preliminary experiments with the Mtw1 kinetochore complex are presented. The consequences of Dsn1 phosphorylation by Hrr25, however, remain undefined and phosphorylation of Mam1 (previously suggested to be critical for monopolin function) is not further investigated.
In summary, the authors could increase the impact of the study by analyzing structure-defined mutants in the Hrr25-Mam1 interface for meiotic defects in vivo. Alternatively, the mechanistic consequences of Dsn1 phosphorylation could be studied further. I feel that for a journal with an emphasis on mechanistic insights, such as EMBO J, this type of extension of the structural biology experiments would be required to support publication.

We agree fully with the reviewer that the manuscript could be significantly strengthened by in vivo experiments to test our findings. We generated a series of mutations to *MAM1*, corresponding to that disrupt the interface with Hrr25 in vitro, and tested these for defects in spore viability. While none of the mutants show as severe a viability phenotype as the *mam1Δ* strain, there are nonetheless severe defects indicative of meiotic chromosome segregation. These are now described on page 7-8 of the manuscript and in Figure 5. These results strongly support the relevance of our crystallographic data to monopolin function.

The reviewer is also correct that the major remaining question in monopolin function is how the complex specifically cross-links and co-orients sister kinetochores. Based on the results presented here, we suggest that Hrr25 is a major player in determining specificity, and that highly localized kinase activity is critical. Our in vitro results suggest that Dsn1 may be an important Hrr25 substrate, while our earlier results suggested Mam1 as another critical substrate. While we are actively pursuing both of these avenues with in vitro and in vivo experiments to: (1) identify phosphorylation sites on each protein, and (2) determine which sites are most important and (3) how phosphorylation of each affects the dynamics of monopolin-kinetochore interactions, these experiments are significantly beyond the scope of the current work.

Additional correspondence (editor) 18 July 2016

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by two of the original referees (see comments below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal!

Before sending a formal letter of acceptance, I would however ask you to briefly respond (simply via email) to the few remaining comments of referee 1. Ideally, please also attach for our internal review some image files with exemplary source data as mentioned in point 2 - this can be in a raw/unpolished format. Regarding point 3, we see no problem with the formatting as all files are labeled as requested and will be properly typeset, but you may want to add additional clarification in response to the second half of point 3.

After that, I expect we should be able to swiftly proceed with formal acceptance and production of the manuscript.

REFEREE COMMENTS

Referee #1

(Report for Author)
The revised manuscript by Ye et al. addresses some issues raised during the initial review. My principle concerns were claims made with regard to the effect of Mam1 on the specificity of the Hrr25 kinase and the relative activity of the Hrr25:Mam1 complex compared to Hrr25 alone. Although neither issue is fully addressed in the revised version, I'm satisfied that the claims made in the manuscript have been modified to be consistent with the available evidence. The addition of the in vivo testing of interface disrupting mutations is welcome and I think the manuscript suitable for publication. However, there are a few minor points that could still be addressed at editorial discretion:

1. I'm surprised that the authors claim they cannot generate enough Mtw1 complex to test Hrr25 stimulation (in response to reviewer 2, point 6). From the gel shown in figure 8a, there appears be a good yield of the Mtw1 complex - is this not sufficient for the ATPase assays?

2. It would be be useful if the authors included some representative raw data from their experiments as a matter of course. I'm pleased that the difference maps for ion assignment have now been incorporated, as requested in my original review. However, there are still absences, for example, the spore viability results, presented in figure 5 just present a single number indicating viability. I would like to have seen at least one example tetrad dissection from the wild-type and one compromised mutant presented somewhere. While these are not required for scientific understanding and I have no reason to doubt the integrity of the data, it is reassuring for a referee to see some original experimental results from which the numbers are generated (though this is just a personal opinion).

3. There are some rather frustrating omissions in the presentation of the revised manuscript. For example, supplemental data are referred to as "supplemental" in the rebuttal, but "expanded view" in the revised manuscript. The figures have no title on them, making it difficult to quickly relate them to the text. Some items used in the figures are not identified in the legend (for example, in the spore viability assay, figure 5B, I assume that "-" in the figure means "totally inviable"? - this is not clear. The Mam1 deletion viability, though referred to in the text is not shown in the figure. Addressing these issues would make the manuscript more legible to readers (and keep reviewers happy).

Referee #3

(Report for Author)
The authors have addressed my main concern regarding the lack of functional in-vivo data that would validate the structural insights. They have generated and analyzed in vivo a number of Mam1

point mutants, compromised to varying degrees in the interaction with Hrr25 in vitro. The mutants show defects in meiosis as judged by decreased spore viability and the severity of the meiotic defect largely correlates with the biochemical interaction defect. This provides evidence that the structurally characterized interface is critical for proper function in vivo. By providing a now nearly complete atomic view of the monopolin complex, the study makes an important contribution to the field. I can support publication in Embo J.

Additional correspondence (author) 19 July 2016

1. I'm surprised that the authors claim they cannot generate enough Mtw1 complex to test Hrr25 stimulation (in response to reviewer 2, point 6). From the gel shown in figure 8a, there appears be a good yield of the Mtw1 complex - is this not sufficient for the ATPase assays?

> While it is true that we can purify enough Mtw1 complex for the assays shown in Figure 8A, this is significantly less than that required for examination of kinase stimulation. For the assays shown in Figure 8A, each reaction contained 9.2 ug of Mtw1 complex. For ADP-Glo ATPase assays, we performed assays in 20 uL volumes, and we would need to reach 2 mg/mL protein concentration to match the highest concentration of casein used in Figure 7A. To match the same *molar* concentration of casein, we would need to reach ~12 mg/mL (as the molecular weight of the intact Mtw1 complex is 148 kDa compared to ~24 kDa for bovine casein). Thus, we would require 40-240 ug of Mtw1 complex for the highest-concentration data point alone, compared to ~10 ug per experiment for the assays shown in Figure 8A.
> Frustratingly, a second contributing factor is the highly unreliable nature of Mtw1 complex expression in *E. coli*. The experiments shown in Figure 8A used Mtw1 complex from an uncommonly high-yielding prep of the complex, performed some years ago, that we have not since been able to replicate despite numerous attempts with different expression conditions, *E. coli* strains, and even codon-optimized genes. We are able to more consistently express and purify an Mtw1 complex lacking the disordered N-terminal tail of Dsn1, but as this is the region most likely targeted by Hrr25, such a complex would not be useful for these assays.

2. It would be useful if the authors included some representative raw data from their experiments as a matter of course. I'm pleased that the difference maps for ion assignment have now been incorporated, as requested in my original review. However, there are still absences, for example, the spore viability results, presented in figure 5 just present a single number indicating viability. I would like to have seen at least one example tetrad dissection from the wild-type and one compromised mutant presented somewhere. While these are not required for scientific understanding and I have no reason to doubt the integrity of the data, it is reassuring for a referee to see some original experimental results from which the numbers are generated (though this is just a personal opinion).

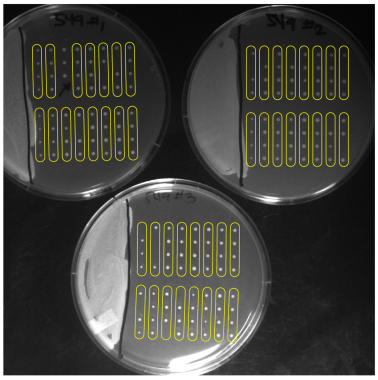
> Please see attached figure file showing raw tetrad dissection data for three strains: wild-type, *mam1Δ,* and *mam1-R149A*. In these images, each dissected tetrad is outlined by a yellow box. Otherwise, the image files have not been

altered. We are happy to include this data in the published manuscript as supplemental/extended view data.
In the dissection of strain KC556 (*mam1-R149A*), several small colonies are visible, often in two of the four spores of the tetrad. These were scored as viable in our analysis, but they likely represent aneuploid products of meiosis.

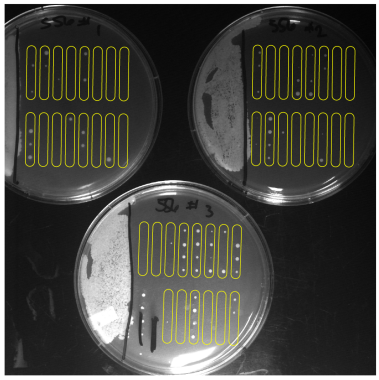
3. There are some rather frustrating omissions in the presentation of the revised manuscript. For example, supplemental data are referred to as "supplemental" in the rebuttal, but "expanded view" in the revised manuscript. The figures have no title on them, making it difficult to quickly relate them to the text. Some items used in the figures are not identified in the legend (for example, in the spore viability assay, figure 5B, I assume that "-" in the figure means "totally inviable"? - this is not clear. The Mam1 deletion viability, though referred to in the text is not shown in the figure. Addressing these issues would make the manuscript more legible to readers (and keep reviewers happy).

We apologize for the confusion regarding “supplemental” versus “expanded view” notation – this was brought on by a very late transition to the “expanded view” terminology in the manuscript proper. The resubmitted figure files were submitted with the intent of making eventual typesetting as easy as possible, hence they lacked anything not to be included in the final figures themselves. For Figure 5B, “--” actually means “not tested” – we will be sure to note this in the final figure legend (we are happy to provide an updated figure legend immediately, or wait for page proofs to make this change). We did not show the *mam1Δ* spore viability in the figure as there is no obvious place to put it in the figure, and also because *mam1Δ* spore viability has previously been reported by several groups (first in Toth…Nasmyth (2000) *Cell* **103**:1155-1168).

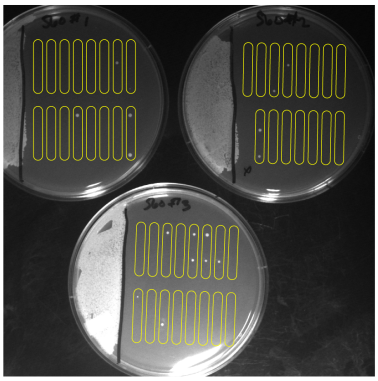
Tetrad dissection for KC549 (wild-type)



Tetrad dissection for KC556 (*mam1-R149A*)



Tetrad dissection for KC560 (*mam1*Δ)



Accepted 20 July 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: Kevin D. Corbett
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2016-94082

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 $\chi 2$ 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).

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
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.	For in vivo studies, strain numbers were used instead of mutant names to obscure the samples expected to have high or low viability.
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?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes
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

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/

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).	NA
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	Database accession numbers are provided in Table S1. In brief, structure factors and ccordinate files are deposited with the RCSB Protein Data Bank under accession codes 4XH0, 4XHG, 4XHH, 4XHL, 5CYZ, and 5CZO. Original diffraction data are deposited in the newly-established SBGrid Data Bank with dataset numbers 151, 152, 153, 154, 195, and 196. All data will be publicly released upon publication.
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	No
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.	No