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Dpb11 coordinates Mec1 kinase activation with cell cycle regulated Rad9 recruitment

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(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 11 May 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. I have now received the reports from three expert reviewers, which you will find copied below. As you will see, the referees acknowledge your comprehensive and thorough analysis, and also appreciate its novel mechanistic implications. At the same time, however, they also raise substantive issues regarding the presentation of the work in the current form, including the lack of discussion of relevant results obtained previously for homologs in the fission yeast system.

Upon deliberation of all comments and further discussions with our Chief Editor, Dr. Pulverer, we decided that we could consider a revised manuscript, thoroughly rewritten in the spirit of the referees comments and addressing also the specific technical issues of referees 2 and 3, further for publication. I would therefore like to invite you to prepare such a revision, taking care to state relevant background information (including the fission yeast references Du et al G&D 2006, Mochida et al EMBOJ 2004, and Furuya et al G&D 2004) already early on in the introduction as well as in the discussion in order to better sculpt out the main novel findings of your own study; at the same time, we would not encourage you to follow referee 1's suggestion of splitting up the paper.

I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to

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grant an extension.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://www.nature.com/emboj/about/process.html

Should you have any further question regarding this decision or your revision, please do not hesitate to contact me. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

There is a vast amount of data in this manuscript. Much of it is a necessary confirmation of what is known in other organisms. It has been very hard for this referee to establish what is new and thus suitable for publication in EMBOJ. For example, the contribution of CDK phosphorylation to the function of the S. pombe homolog of Rad9 has been well characterised in a number of papers from the Russell lab (some of which are not referenced in this manuscript, for example Nakamura 2005) and the function of homologs of Dpb11 as a scaffold in linking the 9-1-1 and ATR-ATRIP branches of checkpoint sensing has been described, although by no means so elegantly, by the Yanagida and Carr laboratories in 2004.
The observation that will be of most interest outside of the budding yeast checkpoint community, and which underpins this manuscript, is mechanistic. Conceptually there is not, in this reviewer's opinion, anything unexpected and the model presented remains consistent with what is predicted from the published literature. Notwithstanding this criticism, there is substantial body of work here which also contributes a significant number of small nuggets of information and allows the authors to draw together a significant number of observations in a single experimental system.

The novel observation: the identification of the residues responsible for the Rad9-Dpb11 interaction as S462 and T474 by phospho-peptide binding, the demonstration that these are phosphorylated using phospho-specific antibodies and the genetic demonstration of functional importance. For many labs this work alone would have been a publishable unit of data. It certainly would have been easier to present and read. A lot of the remaining information in the manuscript is only tangentially related, for example it further narrows down the AAD sequence described and characterised by the Burgers lab and uses a range of elegant genetic approaches to tease out information concerning the previously described interactions between the TopBP1 homologs, ATR homologs and 9-1-1 complex. These additional observations are interesting to the field, but only loosely associated with the main work. While integrating so much information in a single experimental system is useful, at present it does not, as the authors rightly acknowledge, yet explain the relationship between the AADs, the activation of ATR and how these can integrated into a model that explains the role of ATR in establishing its own activation complex.
The experiments are in general beautifully executed. Because the manuscript is complex and contains so much detail, it is hard to penetrate, even for a reviewer familiar with the checkpoint literature. This is a consequence of the choice to present such breadth of data in a single manuscript, and I cannot see how this can be rectified without splitting the work into separate manuscripts - potentially one dealing with the mechanism of Rad9-Dpb11 interactions and a second concentrating on the relationship to Mec1.

Page 4. Alternative modes of Rad9 recruitment. The work of Du et al should be discussed. In this 2006 paper the authors demonstrated parallel recruitment pathways for the Rad9 homolog of S. Pombe via Histone modifications and a CDK phosphorylation-dependent recruitment via the Dpb11 homolog, one of the central themes of this manuscript.

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Referee #2

In this manuscript, Pfander and Diffley convincingly show that the checkpoint mediator protein Rad9 associates with Dpb11 in a phosphorylation-dependent manner. The Rad9-Dpb11 interaction requires two specific CDK phosphorylation sites in Rad9 and the N terminal BRCT repeats of Dpb11. This cell cycle-regulated binding of Rad9 to Dpb11 allows Mec1, which binds to and is stimulated by the C terminus of Dpb11, to efficiently phosphorylate Rad9 in G2 but not in G1. The experiments in this study are well designed and executed, and the model proposed is elegant. However, there are a few important details in the experiments and the model that need some clarification.

Specific comments:

1. Fig. 2I suggests that the Dpb11-Ddc1 interaction is required for the stimulation of Mec1 by Dpb11 C terminus and another Dpb11 function independent of its C terminus (as hypothesized on page 9). If this second function of Dpb11 is mediated by the Dpb11-Rad9 interaction, the rad9 ST464,472AA dpb11Δc mutant should behave identically to the ddc1 T602A mutant in the presence or absence of Dot1. This possibility should be tested.

2. The functional relationship between the Dpb11-Rad9 and Dpb11-Mec1-Ddc2 interactions is somewhat confusing. The biochemical and genetic data in Fig. 5 strongly suggest that these interactions must work together. Dpb11 needs to simultaneously interact with Mec1-Ddc2 and Rad9 to activate checkpoint. However, the data in Fig. 2I led to the hypothesis that the Dpb11-Rad9 interaction may have a function independent of Dpb11 C terminus.

3. Given the redundancy between Dpb11 and Ddc1 in Mec1 stimulation, the model doesn't clearly explain why Dpb11 C terminus is functionally important. In G2, the dpb11ΔC mutant should be able to simultaneously interact with Mec1-Ddc2 (via Ddc1) and Rad9. Ddc1 should be able to activate Mec1-Ddc2 in this complex.

4. The experiments using Rad9-AA-Dpb11ΔN need a few additional controls. Since the BRCT I and II are required for the Dpb11-Sld3 interaction, do cells expressing Rad9-AA-Dpb11ΔN undergo normal DNA replication? If the authors wish to argue that the fusion between Rad9 and Dpb11 bypasses the need for Rad9 phosphorylation and the BRCT I and II of Dpb11, the Dpb11ΔN mutant should be used as a control in Figs. 4C-E.

5. The data in Fig. 4E suggest that Rad9-AA-Dpb11ΔN may be a gain-of-function mutant. Lane 6 suggests that even when Dpb11 is not recruited to Ddc1 (T602A) and the Dot1 pathway is lost, Rad9-AA-Dpb11ΔN is still functional. In contrast, endogenous Dpb11 is clearly not functional in the same genetic background (lane 3). Furthermore, lanes 6 and 9 suggest that in Rad9-AA-Dpb11ΔN expressing cells, Dpb11 C terminus is functional even in the absence of the Dpb11-Ddc1 interaction. This contradicts Fig. 2I's conclusion that the Dpb11-Mec1 interaction is dependent upon the Dpb11-Ddc1 interaction. Some additional analysis of the Rad9-AA-Dpb11ΔN protein may help to clarify the issue.

6. If Rad9 binds H3 K79me and/or pH2A directly, the Rad9-Dpb11 interaction may contribute to checkpoint activation by recruiting Dpb11 to sites of damage. Does the Rad9 TS464,472AA mutant affect the recruitment of Dpb11 to DNA damage sites?

Referee #3

Dpb11 coordinates Mec1 kinase activation with cell cycle regulated Rad9 recruitment. by Pfander and Diffley

When cells are exposed to DNA damaging agents, they arrest the cell cycle through the checkpoint

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signal transduction pathways. Budding yeast Dpb11, a counterpart of human TopBP1, constitutes redundant pathways with Dot1 for the damage checkpoint while it works also for DNA replication. This protein has two pairs of tandem BRCT repeats, which bind to phosphopeptides, and C-terminal extension. The authors found three checkpoint proteins, Mec1, Ddc2 and Rad9 as the proteins binding specifically to Dpb11 and examined the role of Dpb11 in the damage checkpoint. Mec1 and Ddc2 bind to the C-terminal extension and the specific amino acid sequence was identified to activate Mec1 kinase. They also found that CDK phosphorylates the two sites of Rad9 and phosphorylated Rad9 binds to N-terminal tandem BRCT of Dpb11. This CDK-dependent binding explains why Dpb11 functions only in G2/M phase for the damage checkpoint in the absence of Dot1. Consistent with this, the Rad9-Dpb11 fusion protein activates the checkpoint even in G1. The authors further demonstrated that simultaneous binding of Rad9 and Mec1/Ddc2 to Dpb11 enhances the checkpoint activation. This result is definitely new and contributes to the field of checkpoints. The experiments shown in the manuscript were carried out carefully and the results are convincing. However, it is a bit wordy. There are many related reports; Cut5/Rad4, a counterpart of Dpb11 in fission yeast, binds to CDK-phosphorylated Crb2, a counterpart of Rad9, and Mec1 is reported to be activated by the C-terminal extension of Dpb11. Although this reviewer basically supports publication of this manuscript in the EMBO Journal, it is recommended to rewrite the manuscript to focus mainly on new findings; enhancement of checkpoint activation by the Dpb11-mediated interaction between Rad9 and Mec1/Ddc2.

(specific points)

1. There are many other interaction proteins of Dpb11 that were not found in this report. The authors' comments on these proteins (Ddc1, Sld2, Sld3 etx) will be appreciated.

2. P.8, The authors showed specific amino acid substitutions of Dpb11. Cortez group reported the similar substitution (Mordes et al, 2008) and claimed that phosphorylation is important. Comments on this will be appreciated.

3. P.11, the first sentence of the last paragraph. This sentence needs more explanation or references for homologues of Rad9.

4. P.12, the second paragraph. The authors described that rad9 ST462, 474AA mutation is "epistatic" to the ddc1 T602A mutant. There are data indicating that both mutations occur in the same pathway but no clear evidence distinguishing between epistatic and hypostatic. The results shown in Supplementary Figure 6 is not clear enough to describe epistasis.

5. There are dpb11ΔC and dpb11Δ600. They might be the same.

6. More detailed description in methods and legends will be appreciated. For example,
i) No description of concentration of phleomycin.
ii) Need more explanation of Figure 5, especially D; What does "phleomycin -" mean? Are they from different samples?
iii) Supplementary Figure 8C, Is Rad9 level the same? The diffused band caused by phosphorylation may give us the impression that the protein level of the wild type is higher than that of the mutant. Brief explanation in the legend is helpful.

7. Typographic errors:
i) p.21, line 7 from the bottom. Andditionally should read Additionally.
ii) p.22, line 8 from the bottom. 300 KOAc might be 300 mM KOAc.
iii) Reference, Granata et al need more information, doi etc.

1st Revision - authors' response 01 August 2011

Reviewer 1

We are very pleased that the referee felt “the experiments are in general beautifully executed”. This reviewer made two specific points. Firstly, our paper improves our understanding of checkpoint

signalling with regard to mechanism, but does not provide a new concept. Secondly, our paper is too dense and we should split the manuscript in two, one addressing the functional interaction of Rad9 and Dpb11, the second focusing on the relationship with Mec1.

It is true that different aspects of the checkpoint function of Dpb11 orthologues have been described in vertebrates and the two yeasts *S. pombe* and *S. cerevisiae*. There are, however, also species differences (e.g. 9-1-1 interacts with BRCT repeats 1 and 2, not 4 and 5 in human cells). Consequently, a deep understanding of the pathway requires knowledge of all the components and interactions in a single system. Together with previous work, our study makes *S. cerevisiae* the first and only species, where all three Dpb11 checkpoint interactions (Rad9, the 9-1-1-complex and Mec1-Ddc2) have been functionally characterized in detail.

The data presented in Fig. 5 shows the power of this approach. In these experiments we demonstrate that a ternary Rad9-Dpb11-Mec1-Ddc2 is required for efficient checkpoint signalling *in* vitro and *in vivo*. Conceptually, this merges the two previously described aspects of Dpb11 function. Dpb11 activates Mec1 and simultaneously it recruits the substrates. Previously, both functional aspects were examined separately - and in different model organisms and different studies. We think that our work thus provides an important step forward for the checkpoint field.

Because our work actually integrates the cell cycle regulated Dpb11-Rad9 interaction with Mec1 activation by Dpb11, we feel these two parts belong together and are necessary for a complete description of Dbp11 function. We agree that our manuscript contains a substantial number of experiments and was probably too dense. We have now moved some data to the supplementary material and rearranged the paper accordingly.

Reviewer 2

We are very happy to see that also this referee liked our work, and comments that experiments are ”well-designed and executed, and the proposed model is elegant”. The referee wanted us to clarify certain details in our model. We addressed this by several new experiments, which we think significantly strengthen our model.

Specific points:

(1) The referee asked us to investigate epistatic relationship of *rad9-ST464,472AA dpb11ΔC* mutants, since a combination of these mutants with *dot1Δ* would be expected to abolish checkpoint activation similar to *ddc1-T602A dot1Δ*. As suggested we constructed the *rad9-ST464,472AA dpb11ΔC dot1Δ* triple mutant and show that the checkpoint is more defective than the *dpb11ΔC dot1Δ* and *rad9-ST464,472AA dot1Δ* double mutants. Rad53 phosphorylation appears completely abolished in this strain similar to the *ddc1-T602A dot1Δ* mutant. The experiment is now included as Supp. Figure 6C.

(2) We agree that the relationship between Dpb11-Rad9 and Dpb11-Mec1 interactions is, indeed, complex and not entirely straight-forward. Our experiments in Fig. 5 suggest that Dpb11 must interact simultaneously with Rad9 and Mec1-Ddc2 in order to mediate full checkpoint activation and our biochemical experiments suggest a mechanistic explanation: in a ternary Dpb11 complex, Mec1 is stimulated to specifically phosphorylate proteins in this complex.

However, double-mutant analysis (Supp. Figure 6C, Figure 2G-H) shows that checkpoint activation is less affected in *rad9-ST462,474AA* (abolishing the Rad9-Dpb11 interaction) and *dpb11ΔC* (abolishing the Dpb11-Mec1 interaction) compared to *ddc1-T602* or *rad9-ST462,474AA dpb11ΔC*. This argues that Dpb11 has a residual but reduced checkpoint function when it can bind one or the other of Rad9 and Mec1. This residual function depends on the remaining binding partner (Mec1 or Rad9, respectively). Currently, we are not sure about the relevance of this residual activity. We are concerned that these Rad9-Dpb11-9-1-1 and 9-1-1-Dpb11-Mec1-Ddc2 sub-complexes are artifacts seen only in mutant backgrounds (or under conditions of very high DNA-damage (Supp. Figure 10)) and may therefore not be relevant for the checkpoint response in wild-type cells. Needless to say, this is difficult to address with genetics. We have included a statement on p. 23-24 to acknowledge this.

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(3) This is an interesting point. The genetics shows that the AADs of Dpb11 and Ddc1 are not redundant, but instead act in separate pathways. Mechanistically, we do not presently understand this. We presume there will be steric constraints that channel each of the AADs into the two different pathways, but at the moment we can only speculate. We consider the possibility that Dpb11-bound Ddc1 may not be able to bind and activate Mec1, since both interactions require the C-terminal domain of Ddc1. We have included a statement on p. 23 to explain this.

(4) The new Supp. Figure 7A shows that replication profiles in FACS analysis of *RAD9-AA-DPB11ΔN* cells released from G1-arrest appear normal. Of course, we cannot rule out some subtle effect on replication, however we note that all of our experiments use G1 or G2/M arrested cells to minimise any indirect effects caused by replication problems. Because DPB11ΔN is completely defective in replication, it must always be 'covered' by some Dpb11 construct containing all 4 BRCT repeats. To assess the checkpoint function of the ΔN mutant, this 'covering' construct must also be checkpoint defective. This, of course, is the Dpb11ΔC mutant. Because of the logical organisation of the paper, the inability of the ΔN and ΔC mutants to complement each other is the key finding of our last experiment (Fig. 5D). One of the implications of this experiment is that the ΔN mutant is completely checkpoint defective. Because it would involve a complete re-organisation of the paper and because ΔN is so completely checkpoint defective, we don't believe this is an important comtrol in Fig.4 and have decided not to change this part of the manuscript.

(5) Rad9-AA-Dpb11ΔN is certainly a gain of function mutant – it allows checkpoint activation during G1 phase without Dot1. It is the reciprocal of the loss of functionRad9-AA mutant which cannot activate the checkpoint even in G2. We believe that the behaviour of these two mutants argues that phosphorylation of these two residues in Rad9 is both necessary and sufficient for checkpoint activation in the Dpb11 pathway. We also believe the Rad9-AA-Dpb11ΔN mutant is a 'hyperactive' allele. As shown in Supp, Fig. 7B, it activates Rad53 at lower phleomycin concentrations than wild type Rad9 even in G2/M. This figure also includes new data showing that this hyperactivity is dominant over the endogenous Rad9 and Dpb11 proteins. Hyperactivity of this mutant is not surprising as the normally weak and regulated interaction between phospho-Rad9 and BRCT1 and 2 has been replaced by an irreversible covalent bond. We think this probably explains much of the behaviour of this mutant.
Given the network of protein-protein interactions involved in checkpoint signaling, it is perhaps not surprising that a covalent fusion might alter the flux of signaling through different subpathways.

(6) Our genetic data as well as published work suggests that the histone-modification dependent pathway for Rad9 recruitment works in a pathway separate to Dpb11 (Figure 2C, 4B, see also (Furuya et al, 2004; Puddu et al, 2008)). Thus, if Rad9 acted upstream of Dpb11, it would have to be recruited to sites of DNA damage by a currently unidentified mechanism. We have tried to test this idea experimentally (New Supplementary Figure 6C). We took advantage of the fact that Dpb11 is phosphorylated by Mec1 after DNA-damage (Puddu et al, 2008). Similar to published data we found that this modification is abolished in a *ddc1-T602A* mutant, but not in the *rad9-ST462,474AA*. The simplest explanation of this experiment is that Dpb11 is recruited via the 9-1-1 complex (as was also concluded by the Muzi-Falconi lab), and that Rad9 acts downstream of Dpb11 recruitment. We have not attempted any cell biology to support this.

Reviewer 3

We are pleased by the overall very positive comments of this referee and that he/ she found our results convincing. However, this referee suggested that we rewrite the manuscript and put a stronger emphasis on our finding that the ternary Rad9-Dpb11-Mec1/Ddc2 complex is crucial for checkpoint signalling. As described in our cover, we have extensively reorganized the paper to emphasise the new findings. We hope the reviewer agrees that this has greatly improved the manuscript.

Specific points:

(1) The referee enquired why other Dpb11-interacting proteins were not found in our Dpb11 pulldown approach. In case of Sld2 and Sld3 the extremely low expression levels combined with the transient nature of their phosphorylation might impede binding of sufficient material to allow mass-

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spectrometric detection. Indeed we can detect binding of Sld3 in a Western blot. In case of Ddc1 (9-1-1) we have to point out that our initial pulldown was conducted using undamaged cells. Since the Dpb11-Ddc1 interaction requires Ddc1 phosphorylation by Mec1, this might easily explain its absence from the pulldown. Moreover, in budding yeast, Ddc1 binding to Dpb11 was observed only in two-hybrid experiments (Puddu et al, 2008; Wang & Elledge, 2002). We have failed to see this interaction in conventional pulldown experiments and suspect that it requires the DNA/chromatin context.

(2) The referee referred to our point mutations in the C-terminus of Dpb11 and mentioned that the Mordes et al would have introduced similar amino acid substitutions (Mordes et al, 2008). We think that there might be a confusion regarding the different mutations, which we would like to clarify. The mutations described in our paper (WG700,701AA; YG735,746AA) abolish interaction with/and activation of Mec1 and these residues are therefore a critical part of Mec1/ATR-activation motifs. The mutations described by Mordes et al. (T731A; T731E) affect an amino acid residue that may be targeted by Mec1-phosphorylation (Mordes et al, 2008). The proposed model that Mec1-phosphorylation of Dpb11 might enhance its ability to activate Mec1 is very interesting, since it might have important implications regarding how the checkpoint signal is amplified.
T731 and YG735,736 are part of one conserved seven amino acid motif, and it is therefore interesting to speculate that phosphorylation might directly affect the interaction of Dpb11 and Mec1. We have therefore included this point in our discussion. Further studies will however be needed to clarify the importance of this putative phosphorylation event for checkpoint signalling *in vivo*.

(3) The referee wondered about our comment that, "clear homologues of Rad9 are present in yeasts and metazoans". To illustrate the diversity of the N-terminal domains of Rad9 homologues, we have now added domain diagrams of budding yeast Rad9, fission yeast Crb2 and human 53bp1 as Supp. Fig. 5b. We are aware that it remains to be demonstrated if 53bp1 is a true orthologue of Rad9 or if some functional aspects are taken over by Mdc1 in metazoa. Therefore we have changed the sentence to: "Homologues of Rad9 that contain conserved C-terminal BRCT and TUDOR domains are found across the eukaryotic kingdom, but upstream of these domains they differ in length and sequence (Supp. Figure 5B)."

(4) We agree with the referee on this point and now changed the corresponding sentence to: "The rad9 ST462,474AA mutation did not lead to an increase in the phenotype of the ddc1 T602A mutant (Supp. Figure 6), which completely abolishes the checkpoint function of Dpb11 providing additional evidence that the rad9 ST462,474AA phenotype is DPB11 dependent."

(5) *dpb11ΔC* and *dpb11Δ600* describe the same mutation, it is now called *dpb11ΔC* throughout the manuscript. We apologize for this confusion.

(6) We have now carefully edited the figure legends and methods to ensure that all necessary information can be found.
ii) "phleomycin –" samples are samples from the same culture as "phleomycin +", but before phleomycin addition.
iii) The referee has correctly pointed out that the level of MBP-Rad9 WT and MBP-Rad9 ST462,474AA in Supp. Fig. 8c is 1.4 fold higher as determined by quantification of the Rad9-Western Blot. Most likely the difference arose during the small scale Cdk-phosphorylation/repurification. We would like to point out that Fig. 5b-c contains equal amounts of MBP-Rad9 and MBP-Rad9ST462,474. We kept Supp. Fig. 8c in the manuscript, since it shows the Dpb11-concentration dependence of Rad9 phosphorylation in addition to what is shown in Fig. 5B-C. We now included a note in the legend of SUP. Fig. 8C in order to point out the difference in protein concentration.

(7) We corrected the typographic errors.

Furuya K, Poitelea M, Guo L, Caspari T, Carr AM (2004) Chk1 activation requires Rad9 S/TQ-site phosphorylation to promote association with C-terminal BRCT domains of Rad4TOPBP1. *Genes Dev* **18:** 1154-1164

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Mordes DA, Nam EA, Cortez D (2008) Dpb11 activates the Mec1-Ddc2 complex. *Proc Natl Acad Sci U S A* **105:** 18730-18734

Navadgi-Patil VM, Burgers PM (2009) The unstructured C-terminal tail of the 9-1-1 clamp subunit Ddc1 activates Mec1/ATR via two distinct mechanisms. *Mol Cell* **36:** 743-753

Puddu F, Granata M, Di Nola L, Balestrini A, Piergiovanni G, Lazzaro F, Giannattasio M, Plevani P, Muzi-Falconi M (2008) Phosphorylation of the budding yeast 9-1-1 complex is required for Dpb11 function in the full activation of the UV-induced DNA damage checkpoint. *Mol Cell Biol* **28:** 4782-4793

Wang H, Elledge SJ (2002) Genetic and physical interactions between DPB11 and DDC1 in the yeast DNA damage response pathway. *Genetics* **160:** 1295-1304

2nd Editorial Decision 29 August 2011

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by the original referees 1 and 2 (see comments below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

Before we will able to send you a formal letter of acceptance, I would just like to ask you for a few minor modifications/amendments as suggested by the reviewers. The easiest way for this would be if you simply sent us a modified text file - including the requested changes as well as a brief Conflict of Interest statement - via email; we would then replace the version in our submission system. If appropriate, you could send a modified Figure 6 (see referee 1's comments) also via email.

After that, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

The revised manuscript is significantly easier to read, although still data dense. The key aspect of this work which will interest the checkpoint community is indeed (as the authors state inthier response) the charcaterisation of the various aspects of TopBP1 functionis a single organism.

Minor comments: page 5 line 7. Clarify the comment "Mec1 and ATR are stimulated by binding to Dpb11 and is homolog TopBP1". Perhaps "Mec1 and its homolog ATR are stimulated by binding to Dpb11 and TopBP1 respectively".

Page 15 bottom line. "was affected stronger" to "was affected more strongly"

Fig6 (rather small). Part A Arrow from mec1 to 9-1-1.

Referee #2

The authors have adequately addressed all the questions that I raised on the

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previous submission. It is appropriate to publish this elegant study in the EMBO Journal. Consistent with the hypothesis of this study, a recent study on human TopBP1 also suggested that TopBP1 functions as a scaffold to facilitate phosphorylation of ATR substrates (Liu et al., 2011 Mol Cell 43:192). Although the mechanistic details are different between yeast and humans, citing the human study may help to strengthen the conclusion of this study.

2nd Revision - authors' response 30 August 2011

Thank you very much for your positive response to our manuscript. We are delighted! Attached please find a modified version of the text and figure 6 (as both photoshop and Adobe pdf - the tiff file was too big to email). We have made all the changes reviewer 1 has suggested - he/she was indeed correct about Fig 6. Reviewer 2 requested we cite a recent paper in Mol Cell. We think this is inappropriate for two reasons. First, this paper was published after our 'final' version was resubmitted. And second, although this work involves TopBP1 and ATR, it describes a mechanisms which is completely unrelated to the mechanism described in our paper, so we feel it is largely irrelevant and would only complicate an already complicated story. We hope you agree.

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