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H2A binds Brc1 to maintain genome integrity during S-phase

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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

22 October 2009

Thank you for submitting your manuscript to the EMBO journal. I am sorry for the slight delay in getting back to you, but in this case it has unfortunately taken a little bit longer than anticipated to receive the reports. I have now received two of the reports, which I have enclosed below. We are still waiting for a third report, but given the present majority recommendation I can make a preliminary decision now to save time. This decision is still subject to change should the third referee offer strong and convincing reasons for doing so. As you can see, both referees find the analysis very interesting, well done and suitable for publication here. They bring up some specific issues as detailed below, which should not involve too much work to resolve. I would therefore like to ask you to start thinking about making the requested changes and additions to the manuscript that would render the paper suitable for publication in the view of these two reviewers. We will forward the comments of the third referee to you as soon as we receive them, together with our final editorial decision.

Yours sincerely,

Editor
The EMBO Journal

REFeree REPORTS

Referee #1 (Remarks to the Author):

In this manuscript, Williams and colleagues present a genetic, biochemical, structural and cell biological analysis of the Brc1 BRCT5-6 domains that strongly support a direct recognition of gamma-H2A by these domains.

The paper is technically outstanding and opens up a number of new areas for exploration. It is a strong candidate for publication.

I have the following comments.

1- It would be important to test whether the Brc1 foci are dependent on Crb2, the only other known gamma-H2A binder.

2- As a follow-up to Figure 4, it would be important to examine whether the brc1-T672A or -K710M alleles are epistatic to the hta-AQ mutation in order to test possibility that the sensitivity of the hta mutant to replication stress is due to a failure to recruit Brc1.

3- The Discussion is really long and it is certainly a section of the paper that would benefit from streamlining. For example, the chromatin-mediated function of Brc1 is incredibly speculative. What is the evidence that "Brc1 appears poised to coordinate the physical proximity of the broken replication fork with the sister chromatid..."? Also how are the results presented suggest in any way, shape or form, that the electronegative patch on the Brc1 BRCT domain promote some sort of nucleosome remodelling? I would delete this section in its entirety.

Referee #2 (Remarks to the Author):

The manuscript describes role of Brc1 protein in DNA repair. Complimentary approaches including genetics, biochemistry, and structural biology were utilized to demonstrate γ H2A-mediated involvement of Brc1 in stalled replication repair, a poorly understood today process. Brc1 foci were observed in response to replication stress and under normal conditions at the chromatin regions prone to replication pause. As predicted, Brc1 was recruited by phosphorylated form of H2A. Comparative studies with other DNA repair and recombination proteins outlined specific role of Brc1 in replication errors processing, rather than in general DSB repair. High resolution crystal structures of H2A binding domain in apo form and in complex with phosphorylated H2A peptide were obtained, and stoichiometry of the complex was addressed with SEC and SAXS methods. The structure provided high resolution picture of interaction and, together with previously reported structures of γ H2A binding BRCT repeats, renders common and specific features of each complex. Structure-guided mutagenesis was utilized to investigate involvement of specific Brc1/ γ H2A interactions in Brc1 recruitment.

Overall, the manuscript provides strong evidence about Brc1 involvement in replication repair both under stressed and normal conditions. The findings are of a great importance and definitely worth publishing.

Data are well presented and illustrated. Thoughtful discussion addresses specific structural features to predict mechanism of Brc1 activity and different modes of interaction with chromatin.

Small comment: "Since Brc1..." sentence on page 10 does not sound right, as well as "biological conditions".

Additional Correspondence

28 October 2009

Sorry for not getting back to you earlier, but I was away from the office yesterday. We have now received the last report on your manuscript and as you can see below this referee is also very supportive of your study. The referee raises some different issues that should be further resolved, but it should not involve too much additional work to address these.

Yours sincerely,

Editor

The EMBO Journal

REFEREE REPORT

Referee #3:

In this study Russell, Tainer and coworkers provide evidence a pair of BRCT domains in fission yeast Brc1 binds to the phosphorylated tail of H2A in an unperturbed cell cycle and after DNA damage. This is supported by *in vivo* imaging of Brc1 foci and by *in vitro* binding studies with peptides and recombinant proteins. The Brc1 foci seen in an unperturbed cycle are thought to correspond to sites of replication fork arrest, possibly rDNA where there is a relatively high concentration of stalled forks. Structural analysis of a Brc1 BRCT pair bound to the phospho-H2A peptide shed light on the mechanism of phospho-recognition. Mutation of key residues involved in binding of Brc1 to phospho-H2A abolish Brc1 focus formation *in vivo* and reduce very slightly the ability of Brc1 to rescue the DNA damage sensitivity of *brc1* cells.

This is a very nice paper that makes a significant step forward in our understanding of Brc1 and the related BRCT domain proteins that have remained somewhat enigmatic. However, based on what is out there in the literature already, this reviewer is somewhat concerned that the model put forward does quite agree with other data on the BRCT domains of Brc1-related proteins. Also some extra experiments to clarify the mechanisms put forward are required. If the authors can address these concerns fully and satisfactorily then this reviewer would be supportive of publication.

1. Deletion of *brc1* in Fig. 1A causes increased Rad22 foci. Is this accompanied by increased breakage at rDNA judged by PFGE?
2. Does the number of Brc1 foci increase in cell with *ts* mutants of replication factors?
3. Do Brc1 foci in an unperturbed cycle colocalize with phospho-H2A foci? This is an important addition to the experiment showing that the *hta-AQ* mutant abolishes Brc1 foci.
4. Does deletion of BRCT 5,6 abolish focus formation of GFP-Brc1 in an unperturbed cycle? Do BRCT 5,6 alone form foci?
5. It is surprising that no epistasis experiments with Brc1 and *hta-AQ* were carried out.
6. It is interesting that the authors point out that residues lining the walls of the Brc1-phosphoH2A interaction pocket are conserved in budding yeast Rtt107 and in mammalian PTIP. But this is also a worry. It has been shown that whereas MDC1 binds to the phosphorylated C-terminal tail of H2AX *in vitro*, PTIP does not (Cell (2005) 123,1213-26). This is not what one would expect if the similarity in the interaction pocket described in this study translates to functional conservation. Have the authors examined binding of PTIP to phosphoH2A peptide themselves? Is not they should not comment that it is likely PTIP binds to phospho-H2AX because they have no valid reason to do so. Although it has been shown that H2AX phosphorylation is likely to be important for PTIP focus formation (after IR) the available data suggest that this most likely reflects an indirect role for H2AX in recruiting MDC1, RNF8 and RNF168 that help recruit PTIP. This paper would be greatly improved if the authors re-examined PTIP binding to phosphoH2A peptide, a negative or positive result would be equally interesting. They could then either comment on functional conservation or alternative mechanism for PTIP.
7. The authors should show the level of expression of the mutants in Fig 3G by western blot.
8. Does HU increase phospho-H2A foci in the experiments in Fig. 5A? It seems

puzzling that H2A foci form at arrested replication forks and these recruit Brc1 but not Crb2 whereas after IR both proteins are recruited to foci. There are possible explanations, they must be discussed.

9. The marker for rDNA was not good and co-localization with Brc1 was not convincing. Can a better marker be used?

1st Revision - authors' response

23 November 2009

Referee #1 (Remarks to the Author):

In this manuscript, Williams and colleagues present a genetic, biochemical, structural and cell biological analysis of the Brc1 BRCT5-6 domains that strongly support a direct recognition of gamma-H2A by these domains. The paper is technically outstanding and opens up a number of new areas for exploration. It is a strong candidate for publication. I have the following comments.

1- It would be important to test whether the Brc1 foci are dependent on Crb2, the only other known gamma-H2A binder.

We have tested this experimentally and now show in Supplementary Figure S1 that spontaneous GFP-Brc1 foci are still formed in crb2Δ cells.

2- As a follow-up to Figure 4, it would be important to examine whether the brc1-T672A or -K710M alleles are epistatic to the hta-AQ mutation in order to test possibility that the sensitivity of the hta mutant to replication stress is due to a failure to recruit Brc1.

We originally thought that genetic epistasis studies of the brc1-BRCT mutations and hta-AQ would be useful in the current analysis. However, initial results suggest these will be complex to analyze and hence should be a separate study. We are in the midst of extensive analysis that will include unpublished BRCT domain alleles of crb2. Given the large amount of new data being generated from these experiments we feel that it would be impractical to include them in the current manuscript.

3- The Discussion is really long and it is certainly a section of the paper that would benefit from streamlining. For example, the chromatin-mediated function of Brc1 is incredibly speculative. What is the evidence that "Brc1 appears poised to coordinate the physical proximity of the broken replication fork with the sister chromatid..."? Also how are the results presented suggest in any way, shape or form, that the electronegative patch on the Brc1 BRCT domain promote some sort of nucleosome remodelling? I would delete this section in its entirety.

We have revised the Discussion to make it more concise. However, it is our stance that the hypothesis for a functional role for the large electronegative surface region of Brc1 acting in a chromatin remodeling function stems from three direct experimental results presented in the manuscript:

- 1) *The Brc1 BRCT-5/6 domains bearing an unusually electronegative patch directly bind to the histone octamer.*
- 2) *Mutations ablating the Brc1-γH2A interaction are hypomorphic, suggesting additional critical functions other than direct tethering to γH2A tail are important for in vivo function.*
- 3) *Macromolecular surfaces are free to evolve considerably unless they are biological interactions regions. However, in this case, the unusual electronegative surface unveiled by the Brc1 X-ray structure is, unexpectedly, structurally conserved with another γH2A interacting protein, hMDC1. This suggests the electronegative surface is functionally critical in these histone-interacting proteins.*

Close juxtaposition and phosphorylation-dependant tethering of this, a large, conserved negatively charged surface, to the positively charged histones core suggests charge-charge interactions may occur. Moreover, in light of remarks from reviewer 2 who comments: "Thoughtful discussion addresses specific structural features to predict mechanism of Brc1 activity and different modes of

interaction with chromatin", we would like to maintain the hypothesis of a potential mode of action that is a direct extension of multiple lines of evidence presented in the results and discussion text.

Referee #2 (Remarks to the Author):

The manuscript describes role of Brc1 protein in DNA repair. Complimentary approaches including genetics, biochemistry, and structural biology were utilized to demonstrate gH2A-mediated involvement of Brc1 in stalled replication repair, a poorly understood today process. Brc1 foci were observed in response to replication stress and under normal conditions at the chromatin regions prone to replication pause. As predicted, Brc1 was recruited by phosphorylated form of H2A. Comparative studies with other DNA repair and recombination proteins outlined specific role of Brc1 in replication errors processing, rather than in general DSB repair. High resolution crystal structures of H2A binding domain in apo form and in complex with phosphorylated H2A peptide were obtained, and stoichiometry of the complex was addressed with SEC and SAXS methods. The structure provided high resolution picture of interaction and, together with previously reported structures of gH2A binding BRCT repeats, renders common and specific features of each complex. Structure-guided mutagenesis was utilized to investigate involvement of specific Brc1/gH2A interactions in Brc1 recruitment. Overall, the manuscript provides strong evidence about Brc1 involvement in replication repair both under stressed and normal conditions. The findings are of a great importance and definitely worth publishing. Data are well presented and illustrated. Thoughtful discussion addresses specific structural features to predict mechanism of Brc1 activity and different modes of interaction with chromatin.

Small comment: "Since Brc1..." sentence on page 10 does not sound right, as well as "biological conditions".

Thank you for identifying these errors. These suggested changes to the text have now been made.

Referee #3 (Remarks to the Author):

This is a very nice paper that makes a significant step forward in our understanding of Brc1 and the related BRCT domain proteins that have remained somewhat enigmatic. However, based on what is out there in the literature already, this reviewer is somewhat concerned that the model put forward does quite agree with other data on the BRCT domains of Brc1-related proteins. Also some extra experiments to clarify the mechanisms put forward are required. If the authors can address these concerns fully and satisfactorily then this reviewer would be supportive of publication.

1 - Deletion of *brc1* in Fig. 1A causes increased Rad22 foci. Is this accompanied by increased breakage at rDNA judged by PFGE?

We have not yet detected increased rDNA breakage by PFGE. It appears that quantitation of Rad22 foci as an indicator of sites of recombinational repair is a more sensitive assay.

2 - Does the number of Brc1 foci increase in cell with ts mutants of replication factors?

*We have not yet examined Brc1 foci in DNA replication mutants; however, some of these DNA replication mutants have strong genetic interactions with *brc1* γ H2A-binding mutants (our unpublished data).*

3 - Do Brc1 foci in an unperturbed cycle colocalize with phospho-H2A foci? This is an important addition to the experiment showing that the *hta-AQ* mutant abolishes Brc1 foci.

GFP-Brc1 foci are visualized in live cells, whereas γ H2A nuclear signals are detected in nuclear spreads (e.g., Zou et al., Mol. Cell. Biol. 25:5363). The two methods are incompatible. However, given the data in Figures 1-3 and Supplementary Figure S2, we believe it is reasonable to conclude that Brc1 foci formation requires direct binding to γ H2A.

4 - Does deletion of BRCT 5,6 abolish focus formation of GFP-Brc1 in an unperturbed cycle? Do

BRCT 5,6 alone form foci?

Brc1 BRCT5-6 is required for Brc1 foci formation, but it is also required for efficient Brc1 nuclear localization. We see hints of foci formation when expressing BRCT5-6 alone, although this protein is found both in the nucleus and cytoplasm. These data are presented in Supplementary Figure S3.

5 - It is surprising that no epistasis experiments with Brc1 and hta-AQ were carried out.

We originally thought that genetic epistasis studies of the brc1-BRCT mutations and hta-AQ would be useful in the current analysis. However, initial results suggest these will be complex to analyze and hence should be a separate study. We are currently in the midst of extensive analysis that will include unpublished BRCT domain alleles of crb2. Given the large amount of new data being generated from these experiments we feel that it would be impractical to include them in the current manuscript.

6 - It is interesting that the authors point out that residues lining the walls of the Brc1-phosphH2A interaction pocket are conserved in budding yeast Rtt107 and in mammalian PTIP. But this is also a worry. It has been shown that whereas MDC1 binds to the phosphorylated C-terminal tail of H2AX in vitro, PTIP does not (Cell (2005) 123,1213-26). This is not what one would expect if the similarity in the interaction pocket described in this study translates to functional conservation. Have the authors examined binding of PTIP to phosphoH2A peptide themselves? Is not they should not comment that it is likely PTIP binds to phospho-H2AX because they have no valid reason to do so. Although it has been shown that H2AX phosphorylation is likely to be important for PTIP focus formation (after IR) the available data suggest that this most likely reflects an indirect role for H2AX in recruiting MDC1, RNF8 and RNF168 that help recruit PTIP. This paper would be greatly improved if the authors re-examined PTIP binding to phosphH2A peptide, a negative or positive result would be equally interesting. They could then either comment on functional conservation or alternative mechanism for PTIP.

In the revised manuscript we note that it is interesting that H2AX-binding residues appear to be conserved in PTIP, and then cite the studies showing that whilst PTIP requires γ H2AX for formation of ionizing radiation induced foci, this is thought to involve an indirect mechanism requiring MDC1 and RNF8. We agree that in light of our results it will be interesting to reassess PTIP binding to γ H2AX using purified BRCT5-6 domains.

7 - The authors should show the level of expression of the mutants in Fig 3G by western blot.

The microscopy images shows that the mutant proteins are nuclear-localized and are not under-expressed relative to wild type. From these data we believe it is reasonable to conclude that the mutant phenotypes result from an inability to bind γ H2A.

8 - Does HU increase phospho-H2A foci in the experiments in Fig. 5A? It seems puzzling that H2A foci form at arrested replication forks and these recruit Brc1 but not Crb2 whereas after IR both proteins are recruited to foci. There are possible explanations, they must be discussed.

The possible mechanisms explaining the differences in Brc1 and Crb2 foci in HU-treated cells were discussed in the section 'Distinct types of γ H2A-dependent Brc1 foci'.

9 - The marker for rDNA was not good and co-localization with Brc1 was not convincing. Can a better marker be used?

This is the best marker for live cell studies. It was used in other studies, most recently in Pebernard et al. 2008 (EMBO J, 27:3011).

Thank you for submitting your revised manuscript to the EMBO Journal. I asked referees # 1 and 3 to review the revised version and I have now heard back from both referees. As you can see below,

there are still some concerns with the present version. The main one is the epistasis analysis that was requested during the initial round of review that has not been addressed. As referee #1 points out, this issue has to be further clarified and satisfactorily addressed before acceptance here. If you can address the remaining issues, including adding data to resolve the epistasis issue, then we are willing to consider a final revision.

Yours sincerely,

Editor
The EMBO Journal

REFeree REPORTS

Referee #1 (Remarks to the Author):

I only had one critical point in my first review, it was point 2:

"2- As a follow-up to Figure 4, it would be important to examine whether the *brc1*-T672A or -K710M alleles are epistatic to the *hta*-AQ mutation in order to test possibility that the sensitivity of the *hta* mutant to replication stress is due to a failure to recruit Brc1."

It is not acceptable to this reviewer to brush this point aside by saying it is "complicated" and the focus of "other studies". The model put forth by the authors suggests that there should be epistasis and if it is more complicated than alluded to in the manuscript, then the authors must clarify the situation.

Referee #3 (Remarks to the Author):

The authors should replace the term "*Brc1*^{superscript}-Rtt107/PTIP" in the 8th line of the abstract with simply Brc1. It is far from clear of Rtt107 is related to PTIP, other than in having BRCT domains. Similarly, Brc1 is quite different from Rtt107 in functional terms.

Also, it is NOT clear that Rtt107, PTIP and Brc1 are really members of a family. The only thing they definitely have in common is multiple BRCT domains. So does Dpb11/TOPBP1, for example, but it is not included (rightly). Brc1 has four N-terminal BRCT domains and two at the C-terminus but PTIP has four C-terminal BRCT domains and two at the N-terminus. So even in terms of BRCT domain organisation, these proteins are different.

The authors can discuss these points in the discussion, but it is premature in the Introduction (e.g. 3rd para of Intro, 1st line) to refer to these proteins as members of the same family. It would be more convenient for the authors if they were members of a family but one must exercise caution.

If these important points are addressed, the paper would be acceptable for publication in EMBO Journal.

2nd Revision - authors' response

14 December 2009

Response to referees:

Referee 1:

We have added the epistasis studies requested by the referee. Supplementary Figure S2 shows that H2A-AQ and Brc1 BRCT domain mutants are genetically epistatic in 4 mM HU and 2 micromolar CPT, but not so in a higher dose of CPT. The data indicate that in response to fork arrest or moderate amounts of fork collapse, the function of the function of γ H2A can be largely or entirely

explained by its recruitment of Brc1, whereas a Brc1 independent γ H2A activity comes into play when there is large-scale fork breakage.

Referee 3:

We have modified the references to Rtt107 and PTIP as suggested by the referee. For Rtt107 we point out that it and Brc1 are members of a of 6-BRCT domain protein family that appears to be conserved in fungi (as stated on the NCBI web page for Brc1). For PTIP we point out it shares structural similarities with Brc1/Rtt107 but it is unknown if they are functionally conserved. This description comports with the recent review by Munoz and Rouse (EMBO Reports, 2009) that is cited in the text. The Rtt107/PTIP superscripts in the abstract are replaced with the following phrase “Brc1... is structurally related to *Saccharomyces cerevisiae* Rtt107 and mammalian PTIP.”