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TopBP1 functions with 53BP1 in the G1 DNA damage checkpoint

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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 07 May 2010

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments to the authors are provided below. As you can see from these comments while referee #1 is not persuaded that the advance and insight provided is sufficient to consider publication here, referee # 2 and 3 are more supportive and find the analysis suitable for publication here pending adequate revisions. However, it is also clear from the reports that further data would be needed to strengthen the findings reported and the concerns are clearly outlined below. Given all the available input, I will go with the recommendation of referees #2 and 3. In other words, if you can address their criticisms in full (and also keep in mind the specific points raised by referee #1), we would consider a revised manuscript. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

In this manuscript the authors show that TopBP1 colocalizes with 53BP1 at sites of DSBs in the G1 phase. They then characterize the domains required for this localization and show that BRCT domains 4-5 are responsible for this distribution pattern. There are several problems with the manuscript. The major issue is that many of the findings presented do not recapitulate a physiological condition. In addition there are several experimental problems that have not been properly addressed such as lack of appropriate controls for the experiments shown. In addition, a role for TopBP1 in monitoring DSB formation is not unexpected. Therefore, the data presented lack significant novelty to justify publication in a journal addressing a general audience and might be more appropriate for a specialized journal.

Specific comments:

-DSBs usually arise during S-phase unless cells are treated with DNA damaging agents such as IR during G1. Therefore it is hard to imagine any physiological role for the checkpoint described here under normal cell cycle conditions.

- The quality of the images is rather poor. High levels of background make difficult to clearly see the foci.

-The experiments about TopBP1 localization have been conducted using truncated versions of TopBP1 overexpressed in cells with normal levels of TopBP1. These experiments should have been done in cells in which TopBP1 levels had been down regulated. It is impossible to understand whether the localization and the effects of the different domains are due to their direct interaction with proteins present on DNA or to the displacement of proteins normally bound to endogenous TopBP1.

- Tetramerization domains have been used to force interactions with endogenous proteins. It is difficult to ascertain whether these interactions take place in vivo under normal circumstances.

-siRNA experiments have been conducted without proper controls. For example no siRNA resistant proteins have been used to rescue the effect of the knockdown of TopBP1, 53BP1, ATM or ATRIP.

Referee #2 (Remarks to the Author):

In this paper, Halazonetis and colleagues have explored how the domains of TopBP1 contribute to its ability to respond to DNA damage throughout the various phases of the cell cycle. The major finding of this paper is that the BRCT domains 4-5 contribute to the co-localization of TopBP1 with 53BP1 in damage foci in the early part of the cell cycle (i.e., GI or 2N cells). They also present evidence that this interaction is important for establishment of the G1 checkpoint. Overall, I believe that this finding is quite significant and would merit publication once the following points are addressed.

Points

1. It is well established in both yeast and vertebrate systems that DSBs in G1 and S/G2 phases experience two different pathways for repair. In G1, nonhomologous end joining (NHEJ) prevails, whereas from S to M homologous recombination (HR) is predominant. It is surprising that authors do not mention this issue at all in the manuscript. In addition, in the abstract, the authors stated that "recruitment of TopBP1 to sites of DNA replication stress was dependent upon BRCT 1-2 and 7-8". However, since they have not investigated replication stress directly (by treating with aphidicolin or hydroxyurea), the wording should be modified. The behavior of TopBP1 and its mutants upon replication stress would be additionally informative. Induction of DSBs in >2N cells must be causing complicated checkpoints resulted from resected DSBs as well as stalled replication forks. The first paragraph of the Results section should also consider this issue in connection with the description of RPA and ATRIP foci.

2. Through deletion mapping studies, the authors found that BRCT 4-5 is essential for co-localization of TopBP1 with 53BP1. Tetramerized repeats of the 4-5 fragment also co-localized with 53BP1. Importantly, a key lysine residue at 704 on BRCT 5, which would be crucial for recognizing a phosphorylated residue of a binding partner, was shown to be essential for this association. In one critical experiment (Figure 5D), the authors showed that TopBP1 deleted for BRCT 4-5 could not establish the G1 checkpoint. Overall, the data are consistent with each other. However, it would be more convincing if the authors used TopBP1-K704A mutant instead of the 4-5 deletion for the experiment in Figure 5D.

3. Based on the K704A mutation work, it appears that there could be phosphorylation involved in the association between 53BP1 and TopBP1. It would be informative to test this idea by using siATM, AT cells, siATR, and so forth.

4. In the yeast and frog systems, TopBP1/Dpb11/Cut5 proteins are required for replication initiation. The authors should discuss this issue in connection with the knockdown of wild-type TopBP1 in Figure 5.

Referee #3 (Remarks to the Author):

Comments to the Authors:

In this manuscript the authors describe a new role for the replication stress protein TopBP1 in the G1 cell cycle checkpoint. They propose that TopBP1 is binding via its BRCT domains 4 and 5 to 53BP1 and may mediate the role of 53BP1 in G1 checkpoint. This is a new finding and very interesting. However, before this manuscript is suitable for publication in EMBO J., the authors have to strengthen their story by addressing following issues.

1) Statistics for the immunofluorescence should be included, especially for the one where reduced TopBP1 foci formation is shown after knock down 53BP1. The control in the immunofluorescence for efficient 53BP1 knock down is also missing.

2) The co-immunoprecipitation studies of 53BP1 and TopBP1 are quite convincing, however, to be able to claim that these proteins play a role together in vivo in the G1 DNA damage checkpoint, mapping of the site in 53BP1 that directly interacts with the TopBP1 BRCT tandem domain is necessary. Knowing the site, the phosphorylated Serine or Threonine residue can be mutated. Reduced TopBP1 foci are expected if indeed it is recruited via direct phosphorylation-dependent binding to 53BP1.

3) If possible, 53BP1 deficient cells could be complemented with the mutant 53BP1 that is no longer able to interact with TopBP1, followed by investigation if the G1 checkpoint is impaired. Thus, the authors not only have to speculate but could directly show that TopBP1 mediates the checkpoint function of 53BP1 in G1.

4) In Figure 3, recruitment of the tandem BRCT domain 4/5 alone to DNA damage is shown but only if it is fused with a tetramerization domain. This is an artificial system, since this domain is not derived from TopBP1, but from 53BP1. Is there a similar tetramerization domain in TopBP1? At least, the experiment should be controlled by using this tetramerization domain fused to another protein fragment to show that the tetramerization domain itself is not recruited to sites of DNA damage.

5) It would be interesting to know if the recruitment of 53BP1/TopBP1 in G1 is H2AX dependent, MDC1 dependent and RNF8 dependent.

Minor:

1) Figure 1: Panel D and E: graphs should be shown in stacked columns instead of clustered column for better understanding.
2) Figure 2: It would be much easier to look at the different microscopic pictures if the deletion

mutants are named with numbers or logical names as (f.i. delta-BRCT-4/5) instead of the actual residues. This I would just write in the schemativ view of the deletion mutants (panel 2A).

1st Revision - authors' response 06 August 2010

Response to Referees

We want to thank the Referees for their constructive comments. In response to these comments, we have performed several new experiments showing that: 1) TopBP1 recruitment to sites of DNA DSBs in G1 cells is dependent on ATM, but not ATR (Figure 4D and Suppl. Fig. 6B); 2) BRCT domains 1−2 and 7−8, but not BRCT domains 4−5, recruit TopBP1 to sites of DNA replication stress induced by hydroxyurea (Suppl. Fig. 5); 3) TopBP1 recruitment to sites of DNA DSBs in G1, but not to sites of DNA replication stress, is dependent on histone H2AX phosphorylation, MDC1 and RNF8 (Suppl. Fig. 8). We also provided new quantitative date on the dependency of TopBP1 focus formation on 53BP1 (Suppl. Fig. 6A) and made numerous other changes to enhance the clarity of the manuscript and to address the questions raised by the Referees. We hope that the Referees will consider that the manuscript has been improved. Our detailed response to the comments of the Referees are listed below.

Response to Referee #1:

Specific comments:

"1. DSBs usually arise during S−phase unless cells are treated with DNA damaging agents such as IR during G1. Therefore it is hard to imagine any physiological role for the checkpoint described here under normal cell cycle conditions."

Actually, cells that exit mitosis with DNA damage activate a G1 DNA damage checkpoint. The work of Tyler Jacks and colleagues, describing the response of p53 in cells treated with mitotic poisons, is highly relevant to this point (Mol. Cell Biol. 18: 1055−1064, 1998).

"2. The quality of the images is rather poor. High levels of background make difficult to clearly see the foci."

We avoid increasing the contrast of the images, so that the signal of the foci can be compared to the background signal.

"3. The experiments about TopBP1 localization have been conducted using truncated versions of TopBP1 overexpressed in cells with normal levels of TopBP1. These experiments should have been done in cells in which TopBP1 levels had been down regulated. It is impossible to understand whether the localization and the effects of the different domains are due to their direct interaction with proteins present on DNA or to the displacement of proteins normally bound to endogenous TopBP1."

This is in part a valid concern. However, the various TopBP1 mutants were not grossly overexpressed. Even in transiently−transfected cells, we focused on the cells that expresed low levels of tagged TopBP1 fusion proteins. The relative distribution of these proteins (foci versus nucleoplasm) was similar to that of endogenous TopBP1 (as is evident in the images). Further, these foci contain a large number of molecules and therefore displacement of endogenous proteins by the ectopically−expressed proteins does not occur. This was evident by monitoring the localization of endogenous TopBP1 in cells expressing ectopic GFP−tagged TopBP1 proteins.

"4. Tetramerization domains have been used to force interactions with endogenous proteins. It is difficult to ascertain whether these interactions take place in vivo under normal

circumstances."

We used ectopic tetramerization domains to study the properties of selected pairs of BRCT domains. The BRCT domain pairs are not recruited efficiently to sites of DNA damage, unless they are forced to homo−oligomerize. Thus, to study the recruitment properties of specific pairs of TopBP1 BRCT domains, we had to fuse them to oligomerization domains.

"5. siRNA experiments have been conducted without proper controls. For example no siRNA resistant proteins have been used to rescue the effect of the knockdown of TopBP1, 53BP1, ATM or ATRIP."

For all siRNAs we show depletion of the endogenous protein by western blot (Suppl. Fig. 7). Further, in every experiment, we monitored depletion of the endogenous protein by immunofluorescence. The critical depletion was that of TopBP1, which we did using three different siRNAs with identical results in each case. Further, we rescued the effect of TopBP1 knockdown using an siRNA−resistant full−length TopBP1 clone (Fig. 5D).

Response to Referee #2:

"In this paper, Halazonetis and colleagues have explored how the domains of TopBP1 contribute to its ability to respond to DNA damage throughout the various phases of the cell cycle. The major finding of this paper is that the BRCT domains 4−5 contribute to the co−localization of TopBP1 with 53BP1 in damage foci in the early part of the cell cycle (i.e., GI or 2N cells). They also present evidence that this interaction is important for establishment of the G1 checkpoint. Overall, I believe that this finding is quite significant and would merit publication once the following points are addressed."

Specific Points:

"1a. It is well established in both yeast and vertebrate systems that DSBs in G1 and S/G2 phases experience two different pathways for repair. In G1, nonhomologous end joining (NHEJ) prevails, whereas from S to M homologous recombination (HR) is predominant. It is surprising that authors do not mention this issue at all in the manuscript."

The referee is right and we now discuss this issue in the last paragraph of the Discussion.

"1b. In addition, in the abstract, the authors stated that "recruitment of TopBP1 to sites of DNA replication stress was dependent upon BRCT 1−2 and 7−8". However, since they have not investigated replication stress directly (by treating with aphidicolin or hydroxyurea), the wording should be modified. The behavior of TopBP1 and its mutants upon replication stress would be additionally informative. Induction of DSBs in >2N cells must be causing complicated checkpoints resulted from resected DSBs as well as stalled replication forks. The first paragraph of the Results section should also consider this issue in connection with the description of RPA and ATRIP foci."

We have now examined the formation of TopBP1 foci in response to agents that directly induce DNA replication stress (hydroxyurea). We can therefore now confirm that recruitment of TopBP1 to sites of DNA replication stress was dependent on BRCT domains 1−2 and 7−8. The new data are shown in Supplementary Figure 5 and 8B. In addition, we clarified the first paragraph of the results section.

"2. Through deletion mapping studies, the authors found that BRCT 4−5 is essential for co−localization of TopBP1 with 53BP1. Tetramerized repeats of the 4−5 fragment also co−localized with 53BP1. Importantly, a key lysine residue at 704 on BRCT 5, which would be crucial for recognizing a phosphorylated residue of a binding partner, was shown to be essential for this association. In one critical experiment (Figure 5D), the authors showed that TopBP1 deleted for BRCT 4−5 could not establish the G1 checkpoint. Overall, the data are consistent with each other. However, it would be more convincing if the authors used TopBP1−K704A mutant instead of the 4−5 deletion for the experiment in Figure 5D."

The experiments shown in Figure 5D are performed with stably−transfected clones. It takes significant effort to identify clones that express physiological levels of TopBP1, as validated by analysis of endogenous and ectopic TopBP1 in the same western blot (as shown in Fig. 5D). While we agree that it would be nice to have analyzed the K704A mutant, the benefit to the manuscript would have been incremental.

"3. Based on the K704A mutation work, it appears that there could be phosphorylation involved in the association between 53BP1 and TopBP1. It would be informative to test this idea by using siATM, AT cells, siATR, and so forth."

We performed this experiment and indeed, depletion of ATM by siRNA and analysis of A−T cells, shows that the colocalization of 53BP1 and TopBP1 in G1 cells is dependent on ATM. These new results are shown in Figure 4D and Suppl. Fig. 6B.

"4. In the yeast and frog systems, TopBP1/Dpb11/Cut5 proteins are required for replication initiation. The authors should discuss this issue in connection with the knockdown of wild−type TopBP1 in Figure 5."

The same is true for human cells (we list the relevant papers in the manuscript). In our study we did not observe inhibition of entry into S phase cells (Fig. 5B, non−irradiated cells). We think that, whereas one molecule of TopBP1 per origin is probably sufficient for origin firing, a much larger number of TopBP1 molecules accumulates at the DNA damage foci in G1 cells and is required for the checkpoint function. Hence, the siRNA depletion can affect the G1 checkpoint without inhibiting firing of DNA replication origins. This is now discussed in the manuscript.

Response to Referee #3:

"In this manuscript the authors describe a new role for the replication stress protein TopBP1 in the G1 cell cycle checkpoint. They propose that TopBP1 is binding via its BRCT domains 4 and 5 to 53BP1 and may mediate the role of 53BP1 in G1 checkpoint. This is a new finding and very interesting. However, before this manuscript is suitable for publication in EMBO J., the authors have to strengthen their story by addressing following issues."

"1. Statistics for the immunofluorescence should be included, especially for the one where reduced TopBP1 foci formation is shown after knock down 53BP1. The control in the immunofluorescence for efficient 53BP1 knock down is also missing."

We include now the requested statistics, which support the conclusion that 53BP1 is required for formation of TopBP1 foci in G1 cells (Suppl. Fig. 6A). We also attach an immunofluorescence control for efficient depletion of 53BP1 (see figure below in this file). In the manuscript we showed that depletion of 53BP1 is efficient, as analyzed by immunoblotting (Suppl. Fig. 7) and we monitored also the efficiency of depletion in every experiment we did.

"2. The co−immunoprecipitation studies of 53BP1 and TopBP1 are quite convincing, however, to be able to claim that these proteins play a role together in vivo in the G1 DNA damage checkpoint, mapping of the site in 53BP1 that directly interacts with the TopBP1 BRCT tandem domain is necessary. Knowing the site, the phosphorylated Serine or Threonine residue can be mutated. Reduced TopBP1 foci are expected if indeed it is recruited via direct phosphorylation−dependent binding to 53BP1."

Identifying the phosphorylated residue in 53BP1 that interacts with TopBP1 BRCT domains 4−5 is an important task, but not within our reach, given the time constraints imposed by the journal for receipt of a revised version. However, we performed experiments demonstrating that the interaction between 53BP1 and TopBP1 is dependent on ATM (Figure 4D and Suppl. Fig. 6B). This result suggests that TopBP1 interacts with a residue in 53BP1 that is phosphorylated by ATM. We hope in the future to identify this residue.

"3. If possible, 53BP1 deficient cells could be complemented with the mutant 53BP1 that is no longer able to interact with TopBP1, followed by investigation if the G1 checkpoint is

impaired. Thus, the authors not only have to speculate but could directly show that TopBP1 mediates the checkpoint function of 53BP1 in G1."

This experiment implies that the site of interaction is mapped in 53BP1. Instead, we show the results with the reciprocal experiment using a TopBP1 mutant that cannot interact with 53BP1 (Fig. 5D). We believe that the proposed experiment and the experiment we performed are to some degree equivalent.

"4. In Figure 3, recruitment of the tandem BRCT domain 4/5 alone to DNA damage is shown but only if it is fused with a tetramerization domain. This is an artificial system, since this domain is not derived from TopBP1, but from 53BP1. Is there a similar tetramerization domain in TopBP1? At least, the experiment should be controlled by using this tetramerization domain fused to another protein fragment to show that the tetramerization domain itself is not recruited to sites of DNA damage."

Actually the tetramerization domain is derived from the leucine zipper of GCN4. 53BP1 also has an oligomerization domain, but using it would have been a poor choice, because then the transfected TopBP1 domains might hetero−oligomerize with endogenous 53BP1 and be recruited via endogenous 53BP1 to sites of DNA damage. We include a control in our study; this is the same fragment with point mutations in the BRCT domains that abolish their abilities to interact with phosphopeptides (see Figure 3C and Suppl. Fig. 4 and 5).

"5. It would be interesting to know if the recruitment of 53BP1/TopBP1 in G1 is H2AX dependent, MDC1 dependent and RNF8 dependent."

We performed this experiment. As expected, recruitment of 53BP1 and TopBP1 in G1 cells is compromised in H2AX−/−, MDC1−/− and RNF8−/− mouse embryo fibroblasts, but recruitment of TopBP1 to sites of DNA replication stress is not compromised. These results are shown in Suppl. Fig. 8.

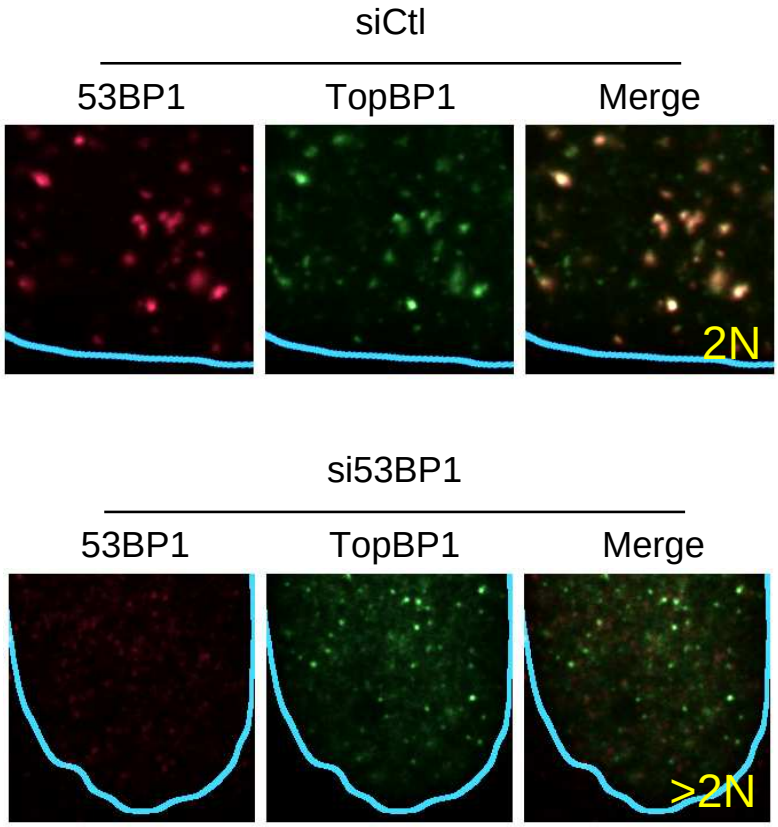
Minor:

"1. Figure 1: Panel D and E: graphs should be shown in stacked columns instead of clustered column for better understanding. 2. Figure 2: It would be much easier to look at the different microscopic pictures if the deletion mutants are named with numbers or logical names as (f.i. delta−BRCT−4/5) instead of the actual residues. This I would just write in the schemativ view of the deletion mutants (panel 2A)."

We made the second change to improve the clarity of the manuscript.

We wish to thank all the reviewers for their constructive comments.

Figure for Referee 3
showing the efficiency of 53BP1 depletion



2nd Editorial Decision 01 September 2010

Thank you for submitting your revised manuscript to the EMBO Journal. Your study has now been seen by the original referees #2 and 3 and their comments to the authors are provided below. As you can see, referee #2 is satisfied with the introduced changes while referee #3 feels that some of the initial raised issues have not been adequately addressed. Referee #3 is nevertheless supportive of publication here given the novelty of the findings and that the manuscript has improved during the revision. Given these comments, I will proceed with the acceptance of the paper for publication here. You will receive the formal acceptance letter shortly.

Sincerely

Editor
The EMBO Journal

REFEREE REPORTS

Referee #2

Overall, the authors have addressed my comments satisfactorily. I believe that the paper is now appropriate for publication in the EMBO Journal.

Referee #3:

In this revised version of their manuscript, Halazonetis and colleagues have adequately addressed some of my previous concerns and criticisms. However, they did not address all of them and therefore, I am still not entirely happy with this manuscript. There are certain points (e.g. the artificial BRCT tetramerization domain) that have to be considered to be dissatisfactory at best (and "shaky" at worst). The authors blame the limited revision time for these shortcomings - a legitimate point in my opinion.

In summary, given the novelty of these findings and the fact that the manuscript has somewhat improved compared to the previous version, I tend to recommend publication in EMBO J,. albeit hesitantly.

