EMBO reports

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CDK targeting of NBS1 promotes DNA-end resection, replication restart and homologous recombination

Jacob Falck, Josep V. Forment, Julia Coates, Martin Mistrik, Jiri Lukas, Jiri Bartek and Stephen P. Jackson

Corresponding author: Stephen P. Jackson, Wellcome Trust and Cancer Research UK, Gurdon Institute

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Editorial Decision:	15 February 2012
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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 15 February 2012

Thank you for your submission to EMBO reports and please accept my apologies because the peer-review process was somewhat longer than would have been ideal given the competitive circumstances. We have now received the enclosed reports from the three referees that were asked to assess your study, referee 2's report is in a different format, as s/he took part in a structured referee report trial. As you will see, all referees are positive about your study and its potential suitability for publication here. Nevertheless, they also have a number of concerns regarding the level of insight and completeness of your results, which would have to be addressed during revision.

As all referees provide constructive suggestions on how to strengthen the work, I am happy to give you the opportunity to revise your manuscript. All referee concerns seem addressable within a reasonable revision time frame. Thus, I think it would benefit the study to attend to all of them. Nevertheless, referee 1's important point 3 and additional point would not be required for publication. If the referee concerns can be adequately dealt with, we would be happy to accept your manuscript for publication. However, please note that it is EMBO reports policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review. As you are in touch with the authors of the competing study, please contact me if timing becomes an issue so we can assess the situation.

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, do not

hesitate to get in touch with me if I can be of any assistance.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

In the manuscript entitled "Cyclin-dependent kinase targeting of NBS1 promotes DNA-end resection, replication restart and homologous recombination" by Professor Stephan Jackson and colleagues the authors discover that NBS1 is phosphorylated in vitro by CDK1/cyclin B, and in vivo by unspecified CDK(s) in S/G2 of the cell cycle. They identify the amino acid target as human NBS1 serine 432, and after mutation of this site provide evidence that phosphorylation is required for efficient IR induction of RPA foci and CHK1 serine 317 phosphorylation, double strand break induced homologous recombination, and recovery from replication stress. However, NBS1 serine 432 phosphorylation is not required for IR induced ATM activation, or for non homologous end joining in a plasmid based assay. This array of phenotypes closely resembles that previously reported for mutational inactivation of the nuclease activities of Mre11. Thus the authors speculate that CDK dependent phosphorylation of NBS1 regulates end resection that is dependent (in part) on the nuclease activities of Mre11.

This manuscript addresses a very important emerging topic - the interface between DNA repair and cell cycle machineries. While it has long been appreciated that DNA repair proteins impact the cell cycle apparatus through induction of checkpoints, the reverse relationship in which core cell cycle kinases regulate DNA repair proteins in the normal cell cycle is relatively new. Specifically, there appears to be an important mechanistic interplay between the CDKs and the MRN complex, along with CtIP.

For the most part, the individual experiments presented by Falk et al are well executed and presented. The manuscript is written clearly, and would be easy to understand by the general readership of Embo Reports. The main criticism is that the study as a whole is quite superficial. In some cases, the limited depth of the study leaves open a chance that the authors conclusions could be proven incorrect when deeper follow up studies are performed. Its seems that this study has been rushed to completion, especially given the previous track record of large, in depth, definitive publications by the Jackson group. However, this reviewer appreciates that time might have suddenly become a factor. Therefore I recommend publication in Embo Reports if the essential points listed below are addressed. Additional points and commentary are provided.

Essential points:
1. The manuscript currently lacks any mechanistic insight to explain why CDK dependent phosphorylation controls MRN dependent resection. The obvious model to test is that this phosphorylation somehow controls CtIP interaction with NBS1. After all, CtIP is a substrate of CDK, interacts with NBS1, and is required for MRN dependent resection in vivo and Mre11 nuclease activity in vitro (much of which was demonstrated by Professor Jackson). The requirement for NBS1 ser 432 phosphorylation in MRN-CtIP interaction can be easily addressed through co-immunoprecipitations. This might not even require new experiments given that immunoprecipitations of tagged NBS1 are presented in fig 1e. Does CtIP co-IP? Even if no impact of the NBS1 mutation is seen, this would be an important observation for the manuscript.
The importance of the relationship between CDK, MRN, and CtIP is emphasized in a manuscript recently published in Nature Structural and Molecular Biology (Buis el at 2012). Apparently, there is an interaction between MRN and CDK that is important for CtIP dependent resection. This highlights the importance of determining the impact of the NBS1 mutation on CtIP. Regardless of the findings, the Buis et al paper should be discussed in the manuscript.

2. An obvious prediction from the authors model is that a phosphomimetic mutation of Serine 432 will permit resection to proceed when CDKs are inhibited. A similar experiment was performed by Huartas and Jackson (JBC, 2009) to demonstrate the importance of CDK control of CtIP dependent resection. At least one important resection assay should be performed with a phosphomimetic NBS1 mutant.

3. P values need to be provided for the bar graphs in Figs 3c and d. This especially applies to d, in which the differences are smaller.

Important points:

1. There is some concern about the analyses in the first few figure panels of the paper. Only CDK1/Cyclin B is tested in vitro in fig 1a, and without controls. The CDKs can be promiscuous, phosphorylating substrates in vitro that are not bone fide substrates in vivo. It would have been nice to see a known substrate and non-substrate tested. Also, another CDK/cyclin could reveal different and important results. Based on the cell cycle profile in figure 1g, CDK2/cyclin A would be a logical choice.
The only real in vivo evidence that CDKs are responsible is the roscovitine incubation in fig 1f. This drug targets CDKs broadly, and has numerous off target effects as well. It would have been nice to see more specific compounds used, as well as RNAi to specific CDKs. Also, some mouse CDK knockout lines are available.

2. In figure 2h, NBS1 phospho serine 432 is reduced 3 hours after ionizing radiation exposure. The authors interpret this as a DNA damage response. However, there is no longer time point to demonstrate the recovery from the response. This leaves open the possibility that reduced NBS1 phosphorylation merely reflects cell death, senescence etc. A longer time point showing recovery is needed.

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Additional point:
It would be nice to see in vitro DNA nuclease assays comparing MRN-CtIP with wt versus mutant NBS1. This would directly address the model put forth by the authors. While this is probably a bit much for a Report, the Jackson lab has taken this approach in the past when studying MRN-CtIP (Sartori et al Nature 2007).

Referee #2:

1. Do the contents of this manuscript report a single key finding? YES

2. Is the main message supported by compelling experimental evidence? NO

If NO, please indicate IN ORDER OF PRIORITY which additional experiments are ESSENTIAL (including controls and statistical analyses, and/or those experiments of low technical quality that must be repeated). Could they be conducted within three months?

Figure 4B shows entry to the M phase following prolonged exposure of cells to HU. This result suggests the following two possibilities, (i) unrepaired DNA damage may activate S and G2 damage checkpoint more strongly in S432A NBS1 expressing cells than in wild-type NBS1 expressing cells, and (ii) wild-type NBS1 expressing cells, but not S432A NBS1 expressing cells, efficiently re-initiated DNA replication, as the authors suggested. To distinguish these two possibilities, the authors should do the analysis shown in Figure 4D, since this analysis selectively analyzes re-initiation of DNA replication excluding the S and G2 damage checkpoint. This experiment could be conducted within a few weeks. I believe that this analysis would increase the value of this manuscript, because replication blockage by aphidicolin followed by very efficient re-initiation of replication promoted by wild-type NBS1 is really interesting.

3. Have similar findings been reported elsewhere (e.g. on a closely related protein; in another organism or context)? NO

4. Is the main finding of general interest to molecular biologists? YES

(1) Regulatory choice of the two major double-strand break (DSB) is the major question in the field.
(2) The critical role of CDK-mediated phosphorylation of CtIP in this regulation was well established. However, it was unclear whether or not additional substrates important for this regulation are present.
(3) This study revealed that NBS1 is another important substrate for CDK mediated promotion of HR.
(4) Moreover, the authors showed compelling evidence for the unexpected conclusion that the phosphorylation of NBS1 by CDK is essential for the re-initiation of DNA replication following replication blockage by aphidicolin and HU.

5. After appropriate revision, would a resubmitted manuscript be most suited for publication:

in EMBO reports

6. Please add any further comments you consider relevant:

1. The ectopic expression of S432A NBS1 and wild type NBS1 restored the X-ray hypersensitivity of NBS1-deficien cells to the same extent (Figure 3B). However, the ectopic expression of S432A NBS1 had no impact on the resection of DSBs (Figure 2D and E) and showed only marginal effects on G2 damage checkpoint (Figure 3A) and HR mediated DSB repair in the artificial construct (Figure 3C). The apparent discrepancy between these defects seen in S432A NBS1-expressing cells and the normal X-ray tolerance of these cells may be attributable to slower proliferation of S432A NBS1 expressing cells in comparison with wild-type NBS1 expressing cells. This is because slower proliferation usually increases cellular tolerance to X-rays. Thus, the manuscript would be improved if the authors add data of cellular proliferation for NBS1-deficient cells and those reconstituted with wild type NBS1 or S432A NBS1.

2. The authors need to describe the incubation time with HU in the legend for Figure 4A, and the concentration of HU in the legend for Figure 4B and C.

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This is a neat paper reporting on the identification of a CDK phosphorylation site in NBS1, Ser 432, which regulates DNA double-strand break repair. DSBs are repaired by non-homologous end joining throughout the cell cycle and by homologous recombination in the S and G2 phases of the cell cycle. Previous studies from the laboratory of Stephen Jackson showed that DNA end resection and homologous recombination is controlled by CDK-mediated phosphorylation of CtIP, a cofactor of MRN.

The authors used a biochemical assay to show that CDK1/CyclinB also targets NBS1 in the MRN complex. They identified a phosphorylation site in NBS1 and generated a phosphospecific antibody to show that NBS1 is phosphorylated in the S and G2 phases of the cell cycle in a CDK-dependent manner. Then they showed that S432A-NBS1 cells fail to form RPA foci and to activate CHK1 when treated with ionizing radiation, suggesting that pS432NBS1 promotes DNA end resection. Consistent with a role for CDK-mediated pS432 NBS1 in regulating DNA repair pathway choice, homologous recombination is impaired and non-homologous end joining is enhanced in S432A-NBS1 cells. Finally, the authors show that pS432 NBS1 is also necessary for recovery from replication arrest.

Overall, the manuscript provides convincing evidence that CDK-mediated phosphorylation of NBS1 controls DSB repair.

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Figure 4B and 4C: the authors should specify in the figure legends and/or in the methods sections the concentration of hydroxyurea and the length of hydroxyurea treatment that is used before release and measurement of pS10-histone H3 positive cells.

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Figure 4D: Hydroxyurea and aphidicolin block DNA replication via distinct mechanisms. Is there any particular reason why the authors used hydroxyurea in Figure 4A, 4B and 4C and switched to aphidicolin in Figure 4D to test replication recovery in S432A NBS1 cells? Is replication recovery similarly impaired following a 6H block in HU?

It is surprising that no CldU incorporation is detected following release from the APH block in S432A NBS1: A number of studies have shown that under stressful conditions, stalled or collapsed replication forks are rescued via the firing of supplementary origins within active replication factories. The authors should perhaps comment on this in there general discussion.

Minor points:
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2) Page 9: The authors judiciously used "replication recovery" rather than "replication fork-restart" in the text and should keep with this terminology in the subtitle "NBS Ser-432 promotes replication-fork restart". The data do not allow distinguishing whether replication recovery occurs via the restart of stalled forks or via the firing of new replication origins.

3) It may be useful to mention in the methods section that readers have access to the anti-NBS1 pSer-432 antibody (abcam).

1st Revision - authors' response 28 March 2012

Referee #1:

In the manuscript entitled "Cyclin-dependent kinase targeting of NBS1 promotes DNA-end resection, replication restart and homologous recombination" by Professor Stephen Jackson and colleagues the authors discover that NBS1 is phosphorylated in vitro by CDK1/cyclin B, and in vivo by unspecified CDK(s) in S/G2 of the cell cycle. They identify the amino acid target as human NBS1 serine 432, and after mutation of this site provide evidence that phosphorylation is required for efficient IR induction of RPA foci and CHK1 serine 317 phosphorylation, double strand break induced homologous recombination, and recovery from replication stress. However, NBS1 serine 432 phosphorylation is not required for IR induced ATM activation, or for non homologous end joining in a plasmid based assay. This array of phenotypes closely resembles that previously reported for mutational inactivation of the nuclease activities of Mre11. Thus the authors speculate that CDK dependent phosphorylation of NBS1 regulates end resection that is dependent (in part) on the nuclease activities of Mre11.

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We thank Referee 1 for his/her overall positive evaluation and for the detailed and insightful comments that he/she has provided. We are pleased that Referee 1 is supportive

of our paper being accepted for publication in EMBO Reports. This referee is correct in his/her assumption that "time is of the essence" – we have learned of complementary work by another group of researchers that is under review for another journal. We are communicating with this other group to try to ensure that one work does not scoop the other.

Essential points:

1. The manuscript currently lacks any mechanistic insight to explain why CDK dependent phosphorylation controls MRN dependent resection. The obvious model to test is that this phosphorylation somehow controls CtIP interaction with NBS1. After all, CtIP is a substrate of CDK, interacts with NBS1, and is required for MRN dependent resection in vivo and Mre11 nuclease activity in vitro (much of which was demonstrated by Professor Jackson). The requirement for NBS1 Ser 432 phosphorylation in MRN-CtIP interaction can be easily addressed through co-immunoprecipitations. This might not even require new experiments given that immunoprecipitations of tagged NBS1 are presented in fig 1e. Does CtIP co-IP? Even if no impact of the NBS1 mutation is seen, this would be an important observation for the manuscript.

We agree with Referee 1 that this is indeed an important point to address. We carried out this experiment and found that mutation of Ser-432 does not prevent the interaction between NBS1 and CtIP. These data are described in the text and presented in Figure 2F of our revised manuscript.

The importance of the relationship between CDK, MRN, and CtIP is emphasized in a manuscript recently published in Nature Structural and Molecular Biology (Buis el at 2012). Apparently, there is an interaction between MRN and CDK that is important for CtIP dependent resection. This highlights the importance of determining the impact of the NBS1 mutation on CtIP. Regardless of the findings, the Buis et al paper should be discussed in the manuscript.

We thank Referee 1 for pointing this out. We did not cite this report before because it was not published at the time of our initial submission. We have cited this work on page 11 of our revised text. For the reviewer's information, we performed some control experiments and noted that CtIP levels and phosphorylation (with or without treatment with DNA-damaging agents) are not detectably affected in NBS cells.

2. An obvious prediction from the authors' model is that a phosphomimetic mutation of Serine 432 will permit resection to proceed when CDKs are inhibited. A similar experiment was performed by Huertas and Jackson (JBC, 2009) to demonstrate the importance of

CDK control of CtIP dependent resection. At least one important resection assay should be performed with a phosphomimetic NBS1 mutant.

NBS1 S432E was indeed produced but we found that it does not work as a phospho-mimetic, constitutively active derivative of the protein in the assays that we carried out. We now note this point in our revised text (last sentence, first paragraph, page 7).

3. P values need to be provided for the bar graphs in Figs 3c and d. This especially applies to d, in which the differences are smaller.

We agree with the reviewer that statistical significance is important in these kinds of assays. We have now provided P values where significance has been found, and have corrected the text accordingly to indicate that there is no statistically significant difference in end-joining efficiency in NBS cells complemented with wild type or S432A-NBS1 (first paragraph, page 9).

Important points:

1. There is some concern about the analyses in the first few figure panels of the paper. Only CDK1/Cyclin B is tested in vitro in fig 1a, and without controls. The CDKs can be promiscuous, phosphorylating substrates in vitro that are not bone fide substrates in vivo. It would have been nice to see a known substrate and non-substrate tested.

Although we agree with the referee that CDKs can be promiscuous in vitro, we think that the fact that Mre11 and Rad50 are not phosphorylated in the same in vitro kinase assay (Fig. 1A) is a good indication of a certain level of specificity.

Also, another CDK/cyclin could reveal different and important results. Based on the cell cycle profile in figure 1g, CDK2/cyclin A would be a logical choice. The only real in vivo evidence that CDKs are responsible is the roscovitine incubation in fig 1f. This drug targets CDKs broadly, and has numerous off target effects as well. It would have been nice to see more specific compounds used, as well as RNAi to specific CDKs. Also, some mouse CDK knockout lines are available.

We agree with the reviewer that roscovitine is a broad CDK inhibitor, and accordingly we have now used specific siRNAs against CDK1 and CDK2 to show that depletion of either of them causes a defect in NBS1 Ser-432 phosphorylation. These new data are shown in Figure 1G of our revised manuscript.

2. In figure 2h, NBS1 phospho serine 432 is reduced 3 hours after ionizing radiation exposure. The authors interpret this as a DNA damage response. However, there is no longer time point to demonstrate the recovery from the response. This leaves open the possibility that reduced NBS1 phosphorylation merely reflects cell death, senescence etc. A longer time point showing recovery is needed.

We feel that it is unlikely that reduced NBS1 phosphorylation could reflect cell death or senescence, and point out that it is also absent in G1 cells (Fig. 1H of our revised manuscript). In addition, the amount of cell killing of SV40 transformed cells after 3 h of being exposed to 5 Gy IR would not account for the difference observed, and of course such cells do not senesce after exposure to DNA damaging agents. We therefore think that it is much more likely to reflect reduced CDK activity, a conclusion that is also supported by the siRNA depletion data for CDK1 and CDK2 in Figure 1G of our revised manuscript (see comments on the previous point raised by this referee).

3. The double strand break repair assays in fig 3c and d entail overexpression of wt or mutant NBS1 in NBS1 deficient cells. This does raise a concern that some findings could differ from experiments performed with NBS1 expressed from the endogenous locus. For example, in fig 3c, would overexpression of wt NBS1 drive HR levels to higher than that in a completely "normal" cell line. If so, how does one interpret the differences in bar graphs when overexpressed. Likewise, could overexpression be masking a subtle End Joining defect in fig 3d? Can the authors validate this approach by showing a control with an NHEJ deficiency that does impact this assay?

Although we understand the reviewer's concerns about over-expression of NBS1, we think that this is unlikely to be a problem due to the fact that the roles of NBS1 in HR and NHEJ also require MRE11 and RAD50 (that is, formation of the MRN complex), and these proteins are not over-expressed. It is thus unlikely that the total amount of active MRN complex in complemented NBS-ILB1 cells differs from that in "normal" cell lines. In any case, recombinant NBS1 levels are very similar between wild type and S432A-NBS1 (see Fig. 1D) and this in our opinion makes the assay valid for comparative purposes.

Additional point:
It would be nice to see in vitro DNA nuclease assays comparing MRN-CtIP with wt versus mutant NBS1. This would directly address the model put forth by the authors. While this is probably a bit much for a Report, the Jackson lab has taken this approach in the past when studying MRN-CtIP (Sartori et al Nature 2007).

In vitro nuclease assays employing MRN and/or CtIP are difficult to perform and interpret because they require the use of highly purified, recombinant proteins that are devoid of contaminating nucleases. Our previous work with such assays relied on highly purified MRN proteins and CtIP kindly provided by the laboratories of Prof. Tanya Paull and Dr. Richard Baer, respectively. In light of these issues and the fact that we are trying to coordinate the publication of our work with a manuscript from other researchers being considered at another journal, we contend that carrying out such nuclease assays is beyond the scope of this current study.

Referee #2:

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2. Is the main message supported by compelling experimental evidence? NO

If NO, please indicate IN ORDER OF PRIORITY which additional experiments are ESSENTIAL (including controls and statistical analyses, and/or those experiments of low technical quality that must be repeated). Could they be conducted within three months?

Figure 4B shows entry to the M phase following prolonged exposure of cells to HU. This result suggests the following two possibilities, (i) unrepaired DNA damage may activate S and G2 damage checkpoint more strongly in S432A NBS1 expressing cells than in wild-type NBS1 expressing cells, and (ii) wild-type NBS1 expressing cells, but not S432A NBS1 expressing cells, efficiently re-initiated DNA replication, as the authors suggested. To distinguish these two possibilities, the authors should do the analysis shown in Figure 4D, since this analysis selectively analyzes re-initiation of DNA replication excluding the S and G2 damage checkpoint. This experiment could be conducted within a few weeks. I believe that this analysis would increase the value of this manuscript, because replication blockage by aphidicolin followed by very efficient re-initiation of replication promoted by wild-type NBS1 is really interesting.

We thank Referee 2 his/her constructive and informed comments and questions. In particular, we thank him/her for the suggested alternative explanation of an M phase delay. We have performed the experiment and the results are now discussed in the new version of the manuscript (results, pages 9-10; discussion, pages 11-12). In brief, our new results indeed indicate that a repair defect rather than a restart defect, as suggested by the reviewer, is the cause of M phase delay in HU-treated cells.

3. Have similar findings been reported elsewhere (e.g. on a closely related protein; in another organism or context)? NO

4. Is the main finding of general interest to molecular biologists? YES

(1) Regulatory choice of the two major double-strand break (DSB) is the major question in the field.
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We understand the reviewer's concerns about proliferation defects, and so we have performed EdU incorporation assays accordingly. The ensuing results are provided in

the new Supplementary Figure S1 (mentioned in the text, page 7), and show no significant differences in EdU incorporation rates between the different cell lines.

2. The authors need to describe the incubation time with HU in the legend for Figure 4A, and the concentration of HU in the legend for Figure 4B and C.

We have now included these data in the revised legend to this figure.

Referee #3:

This is a neat paper reporting on the identification of a CDK phosphorylation site in NBS1, Ser 432, which regulates DNA double-strand break repair. DSBs are repaired by non-homologous end joining throughout the cell cycle and by homologous recombination in the S and G2 phases of the cell cycle. Previous studies from the laboratory of Stephen Jackson showed that DNA end resection and homologous recombination is controlled by CDK-mediated phosphorylation of CtIP, a cofactor of MRN.

The authors used a biochemical assay to show that CDK1/CyclinB also targets NBS1 in the MRN complex. They identified a phosphorylation site in NBS1 and generated a phosphospecific antibody to show that NBS1 is phosphorylated in the S and G2 phases of the cell cycle in a CDK-dependent manner. Then they showed that S432A-NBS1 cells fail to form RPA foci and to activate CHK1 when treated with ionizing radiation, suggesting that pS432NBS1 promotes DNA end resection. Consistent with a role for CDK-mediated pS432 NBS1 in regulating DNA repair pathway choice, homologous recombination is impaired and non-homologous end joining is enhanced in S432A-NBS1 cells. Finally, the authors show that pS432 NBS1 is also necessary for recovery from replication arrest.

Overall, the manuscript provides convincing evidence that CDK-mediated phosphorylation of NBS1 controls DSB repair.

We thank Referee 3 for his/her overall positive evaluation and for the detailed and insightful comments that he/she has provided.

There are, however, a few points that the authors need to clarify:

Figure 4B and 4C: the authors should specify in the figure legends and/or in the methods sections the concentration of hydroxyurea and the length of hydroxyurea treatment that is used before release and measurement of pS10-histone H3 positive cells.

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I was a little surprised not to see a western blot probing for pS4/8-RPA and pS317-CHK1 following release from the HU block (0, 4, 8, 12 and 16 hours after release). The persistence of DNA lesions and of CHK1 activation due to a DNA repair defect could readily explain why S432A NBS1 cells fail to enter mitosis after treatment with HU.
Figure 4D: Hydroxyurea and aphidicolin block DNA replication via distinct mechanisms. Is there any particular reason why the authors used hydroxyurea in Figure 4A, 4B and 4C and switched to aphidicolin in Figure 4D to test replication recovery in S432A NBS1 cells? Is replication recovery similarly impaired following a 6H block in HU?

We thank the reviewer for suggesting this alternative explanation for the defects observed after HU treatment. To help clarify things, the experiment performed with aphidicolin in Figure 4 was also carried out with HU. The ensuing results are now described in the revised main text (results, pages 9-10; discussion, pages 11-12) and indeed point towards a repair defect rather than a restart defect in the case of HU. Aphidicolin was used in the first instance because an earlier report described replication restart defects in NBS cells treated with aphidicolin (Stiff et al, EMBO J 2004).

It is surprising that no CldU incorporation is detected following release from the APH block in S432A NBS1: A number of studies have shown that under stressful conditions, stalled or collapsed replication forks are rescued via the firing of supplementary origins within active replication factories. The authors should perhaps comment on this in there general discussion.

We have now included a discussion on this matter on page 12, first paragraph: "Replication fork collapse under these conditions could result in activation of the intra-S phase checkpoint (mainly via ATR/CHK1) that would prevent rescue of collapsed forks by firing of otherwise dormant, neighboring replication origins (Paulsen and Cimprich, 2007)".

Minor points:
1) Page 7, lane 7 from top: the original article describing the BrdU-based ssDNA detection procedure should be cited: Raderschall E, Golub EI, Haaf T. Nuclear foci of mammalian recombination proteins are located at single-stranded DNA regions formed after DNA damage. Proc Natl Acad Sci U S A. 1999 Mar 2;96(5):1921-6.

We thank Reviewer 3 for pointing the original reference, which we have now included in our revised manuscript.

2) Page 9: The authors judiciously used "replication recovery" rather than "replication fork-restart" in the text and should keep with this terminology in the subtitle "NBS Ser-432 promotes replication-fork restart". The data do not allow distinguishing whether replication recovery occurs via the restart of stalled forks or via the firing of new replication origins.

We thank Reviewer 3 for spotting this oversight; the text has been amended accordingly.

3) It may be useful to mention in the methods section that readers have access to the anti-NBS1 pSer-432 antibody (abcam).

This information has now been added in the supplementary methods section.

2nd Editorial Decision 13 April 2012

I have now heard back form the three referees who assessed your study, who all recommend publication with no further comments. I am thus very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Many thanks for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Editor
EMBO Reports

