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ATR activation and replication fork restart are defective in FANCM-deficient cells

REBEKKA SCHWAB, ANDREW BLACKFORD, WOJCIECH NIEDZWIEDZ

Corresponding author: Wojciech Niedzwiedz, WIMM/OXFORD UNIVERSITY

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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 17 September 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. Your manuscript has now been evaluated by three referees and I enclose their reports below. As you will see from their comments the referees are, in general, positive regarding the study and would potentially be supportive of publication dependent on satisfactory additional experimental analysis. This includes further analysis of and support for the role of FANCM in regulating replication fork dynamics and activation of ATR and phosphorylation of ATR substrates. Should you be able to address these issues, we would be wiling to 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 you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments.

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 study, Schwab and co-workers report on a role for FANCM in controlling replication fork stability/restart and facilitating activation of the ATR signalling pathway following replication stress. The authors utilize DNA fiber analysis to monitor replication fork rates in Wt and FANCM deficient chicken DT40 cells. In asynchronous conditions both cell lines exhibited very similar fork progression rates. However, it was noted that FANCM deficiency resulted in increased origin firing, fork terminations and fork collapse. Consistent with increased fork instability it was shown that FANCM deficient cells exhibit increased levels of spontaneous DNA damage, as marked by gamma-H2AX. Together, these data indicate that replication forks are unstable and more prone to stalling and collapse in the absence of FANCM. The authors then proceed to analyze replication fork integrity in Wt and FANCM deficient cells following treatment with camptothecin (CPT). Although both cell lines exhibited a 2-fold reduction in fork rates, only FANCM deficient cells exhibited a significant increase in new replication initiation events. Analysis of replication fork restart after CPT treatment revealed a striking difference in fork recovery between Wt and FANCM deficient cells; 60% of forks failed to recover in the Wt, whereas only 25% of forks failed to recover in the absence of FANCM. The authors show that this difference is attributed to an increasing in firing of neighbouring dormant origins in the absence of FANCM. Consistent with a role for FANCM in responding to replication stress it was shown that loss of FANCM compromises Chk1 phosphorylation after replication stress. Treatment of Wt cells with inhibitors of Chk1 (UCN-01) was also found to result in increased late origin firing, similar to that seen in FANCM deficient cells. In contrast, UCN-01 treatment did not further increase late origin firing in the absence of FANCM. These data indicated that the increased late origin firing seen in FANCM deficient cells is due to a failure to efficiently active Chk1, which normally suppresses late origin firing. Interestingly, it is shown that caffeine treatment and inhibitors of Plk1 suppressed late origin firing in FANCM cells, whereas ATM inhibition did not. The authors conclude that the increased fork recovery seen in FANCM cells is most likely invoked by ATR and Plk1, but is independent of Chk1. To further investigate the role for FANCM in ATR activation, the authors also analyzed Smc1 S996 phosphorylation after CPT-treatment, which was significantly impaired in the absence of FANCM. It was also shown that chromatin associated RPA is significantly elevated after CPT in the absence of FANCM. In contrast, chromatin associated TopBP1, which is required for ATR activation, was significantly reduced in FANCM cells. The authors conclude that the defect in Chk1 and Smc1 phosphorylation in the absence of FANCM results from a failure to recruit TopBP1, which is required for ATR activation. Finally, the authors show that the role for FANCM in facilitating replication fork recovery and activation of the ATR pathway following replication stress is independent of the FA pathway as DT40 cells deficient for FANCI or FANCC exhibited similar levels of fork recovery and origin firing to Wt cells.

Overall, this is a very interesting and compelling study that builds on previous work in mammalian cells. The results are clear and the conclusions made are full supported by the data. This work will be of great interest to the FA, repair and replication fields.

I have only minor comments/suggestions:

1. The authors show a low basal level of Chk1 phosphorylation in FANCM deficient cells, which is consistent with their findings that these cells also show an increased propensity for fork stalling and/or collapse. Given that Chk1 Ser345 activation (which is triggered by fork stalling/collapse) also targets Chk1 for degradation it is possible that the reduced Chk1 phosphorylation in FANCM cells after CPT treatment reflects low levels of Chk1 due to increased turnover. A similar scenario has been seen in HCLK2 deficient. To address this the authors simply need to provide a total Chk1 blot in Figure 3A.
2. Is the fork remodelling activity of FANCM required for TopBP1 loading onto chromatin? The authors could address this important question by analyzing TopBP1 in FANCM knockout cells expressing the ATPase dead version of FANCM (deficient for fork remodelling).

Referee #2 (Remarks to the Author):

In this manuscript, the authors addressed the role of FANCM, one of the FA genes, during replication fork recovery using DT40 cells as experimental model. Several reports have indicated that FANCM plays important roles in establishing the S-phase checkpoint and favoring either recombination-mediated fork recovery or direct handling of stalled forks. In this context, this manuscript expanded the knowledge on the possible function of FANCM in the response to perturbed replication and provide a detailed analysis of fork dynamic in FANCM-ko cells.
Analysis of replication fork dynamic and checkpoint activation after CPT-induced replication fork damage lead the authors to conclude that FANCM is required to overcome stalled replication forks, recruit TopBP1 to chromatin and sustain CHK1 activation. As a consequence, the authors observe increased firing from dormant origins, probably due to PLK1 activity, which allow completion of replication.
The question of how cells overcome replication stress and maintain proliferation while keeping at low levels genome instability is crucial for basic biology and for the understanding of cancer development. From this point of view, the study of Schwab and colleagues is interesting and provide novel data about the function of FANCM in response to perturbed replication. However, the work requires additional effort to support the findings reported about the relationship between FANCM deficiency, CHK1 activation and dormant origin firing opposed to enhanced recombination as a way to complete replication .
In particular, the authors should include additional experiments analyzing replication fork dynamic after additional labeling periods and perform experiments to assess if CHK1 is simply not activated or rather inactivated by degradation because of PLK1 activity, also addressing whether this function of FANCM is required to deal with HU-mediated replication arrest. Such experiments could contribute to clarify some discrepancies between the reported data, such as low levels of chromatin-bound RPA in wt cells despite the ATR-CHK1 pathway is on.

Specific comments:

Fig. 1. The authors show that FANCM-ko cells have more phosphorylated H2AX than wt and attribute such elevated DNA damage to increased collapse of replication forks under unperturbed replication. This might be correct in theory, however, it is quite surprising that DNA fiber analysis does not detect an increased fraction of stalled forks in FANCM-ko cells. One could envisage that H2AX is not labeling collapsed forks (i.e. DSBs) but fork stalling or some post-replication repair of gaps by HR. Indeed, analysis of RAD51 foci might not detect enhancement of RAD51-independent recombination. To clarify these points, the rate of elongation should be analyzed at shorter and longer labeling times with CldU and double-labelling experiments should be employed to assess whether H2AX co-localizes with replication sites. Please include reference bar for the DNA fibers images.

Fig. 2b,c, d. The authors say that loss of FANCM determines inability to restart replication from damaged forks but a faster recovery because of enhanced firing from adjacent origin. However, I have some problem with these experiments. First of all, from the images of DNA fibers obtained in FANCM-ko cells, it seems that the incorporation of IdU is less efficient (red tracks are longer in the wt). Is this a labeling defect or actually loss of FANCM results in a reduced ability to overcome CPT-induced fork stalling? To address this issue, the authors should adopt a different experimental scheme of IdU labeling. Second, even though I am pretty convinced by the roscovitine experiments that firing of new origins is used by FANCM-ko cells to complete DNA replication, I think that additional experiments using at least one shorter and one longer CldU labeling time should be included to confirm actual firing from adjacent origin and to make comparison with recovery in wt cells. Please, include in the figure legend details about the X-axis of the graphs showed in panel "a".

Fig. 3. No conclusion can be drawn without data on total CHK1 levels. From the blot, one could equally conclude that CHK1 gets activated early after CPT addition and then is deactivated. In view of the findings presented later on in the manuscript, CHK1 degradation could also occur to relieve checkpoint arrest.

Fig. 4. The authors should give a rationale for the choice to check SMC1 phosphorylation as additional ATR target. Most importantly, the results about RPA association with chromatin in wt cells are not consistent with ATR-CHK1 activation. The fractionation protocol used by the authors is very selective and could enrich for proteins that strongly associate with chromatin because, at the

best of my experience, a lot of chromatin-bound proteins dissociate at 420mM of NaCl. To make firm conclusions on ssDNA formation, RPA binding and so on, I advice the authors to repeat experiments analyzing also the 420mM fractions. The same applies to TopBP1 chromatin association.
I will not emphasize the deficient ATR activity in FANCM-ko cells because this point could be demonstrated only performing kinase assays using immunopurified ATR. One minor comment: it is quite expected that levels of DSBs are the same in the two genotypes since DSBs form by collision with the replisome. Any difference should stand in the repair of those DSBs.

Fig. 5. It is not possible to affirm that MCM2 is phosphorylated from the blot presented in the panel "a".

Minor comments: Discussion could be improved by avoiding the lengthy introduction-like paragraphs at the beginning of the section.

Referee #3 (Remarks to the Author):

The authors of this manuscript address the role of FANCM in normal and perturbed S-phase. They show that FANCM has a novel function distinct from the FA complex by demonstrating that it promotes restart of impaired replication forks, and quite intriguingly, that it limits the accumulation of RPA-ssDNA accumulation, a marker for perturbed replication forks. They are able to show by convincing genetic evidence obtained in DT40 cells that this process is controlled by ATR and Plk1, central players in genome stability maintenance pathway. In particular they show that in the absence of FANCM impaired replication forks are unable to restart and that replication completion is ensured by compensatory activation of neighbour origins, whose firing is not affected by FANCM deficiency. This implicates a direct role of FANCM only for the restart of impaired forks. This referees has very much appreciated the effort to analyse and classify all types of replicated molecules by DNA fiber analysis, which allowed correct interpretation of data that would otherwise not be evident.
Through this approach the authors showed that replication origin firing requires Plk kinase and MCM phosphorylation, validating at genetic level previous biochemical findings obtained by other groups. They also clarify the molecular role of FANCM proposing a role for TopBP1 recruitment onto chromatin and consequent ATR activation. I think this is a nice and original piece of work. The data are logically presented and the experiments are well performed. Most of the data make sense with previous results from other groups. There are few discrepancies that should be addressed, in particular regarding the role of FANCM in ATR activation, before publication is granted.

Critiques:

1) The authors claim that ATR cannot be activated in the absence of FANCM. In support of this they show that there is no Chk1 phosphorylation in Fig 3a post CPT. However, they show in Fig 5a that MCM is phosphorylated and they speculate that this event is ATR dependent. I am a bit puzzled by the way the data have been presented. Is ATR responsible for direct MCM phosphorylation in these conditions? The authors could address this point by performing the same experiment of Fig 3a in the presence of Caffeine. If the phosphorylation disappears they might speculate that ATR is directly responsible for MCM phosphorylation in FANCM depleted cells.

2) They authors should better discuss the role of FANCM in promoting Chk1 and Smc phosphorylation. From the data presented in which MCM phosphorylation appears to be intact it seems that FANCM is not promoting the activation of ATR but is involved in substrate selection. It is possible that FANCM is holding the fork structure in a permissive state for Chk1 recruitment and phosphorylation. Consistent with this residual TopBP1 binding present in FANCM -/- cells (Fig 4c) would be sufficient to promote local activation of ATR but to enough to ensure Chk1 activation.

3) What is the impact of Plk inhibitors on survival of FANCM -/- cells in unperturbed and damaged conditions? This is an important point to address the physiological significance of these findings

1st Revision - Authors' Response 04 November 2009

REVIEWER 1

General Comments:
Overall, this is a very interesting and compelling study that builds on previous work in mammalian cells. The results are clear and the conclusions made are fully supported by the data. This work will be of great interest to the FA, repair and replication fields.

Our reply: We thank this reviewer for the positive comment that our manuscript and the data presented therein are solid.

Specific Comment 1: *The authors show a low basal level of Chk1 phosphorylation in FANCM deficient cells, which is consistent with their findings that these cells also show an increased propensity for fork stalling and/or collapse. Given that Chk1 Ser345 activation (which is triggered by fork stalling/collapse) also targets Chk1 for degradation it is possible that the reduced Chk1 phosphorylation in FANCM cells after CPT treatment reflects low levels of Chk1 due to increased turnover. A similar scenario has been seen in HCLK2 deficient. To address this the authors simply need to provide a total Chk1 blot in Figure 3A.*

Our reply. We agree with the reviewer's comment. The point has been addressed and the figure has now been modified as suggested (we included the total Chk1 blot and replaced old Figure 3A with new Figure 3A). The total Chk1 level suggests that in FANCM deficient cells Chk1 turnover is not affected. This has been also verified by analysis of Chk1 stability in FANCM-deficient cells in the presence of cyclohexamide (data not included in the manuscript).

Specific Comment 2: *Is the fork remodelling activity of FANCM required for TopBP1 loading onto chromatin? The authors could address this important question by analyzing TopBP1 in FANCM knockout cells expressing the ATPase dead version of FANCM (deficient for fork remodelling).*

Our reply: We performed additional experiments to address this important issue. To this end we have used the FANCM walker box B point mutant cell line (ATPase dead) as described in Rosado et al. Nucleic Acids Research, 2009. The new data indicate that the helicase activity of FANCM is required for TopBP1 loading onto chromatin. We have also revised the main body of the manuscript to account for the new data (Fig. 4C).

REVIEWER 2

General Comments:
The question of how cells overcome replication stress and maintain proliferation while keeping at low levels genome instability is crucial for basic biology and for the understanding of cancer development. From this point of view, the study of Schwab and colleagues is interesting and provide novel data about the function of FANCM in response to perturbed replication. However, the work requires additional effort to support the findings reported about the relationship between FANCM deficiency, CHK1 activation and dormant origin firing opposed to enhanced recombination as a way to complete replication.
In particular, the authors should include additional experiments analyzing replication fork dynamic after additional labeling periods and perform experiments to assess if CHK1 is simply not activated or rather inactivated by degradation because of PLK1 activity, also addressing whether this function of FANCM is required to deal with HU-mediated replication arrest. Such experiments could contribute to clarify some discrepancies between the reported data, such as low levels of chromatin-bound RPA in wt cells despite the ATR-CHK1 pathway is on.

Our reply: We are very pleased that the referee notes the potential of our paper.
We agree with the reviewer's comments and to address his/her concerns we performed additional experiments - as suggested by the reviewer. We feel that the new data strengthen our initial observation that the recovery of DNA synthesis in DFANCM cells indeed involves dormant origin firing.

For the benefit of the reviewer, here are, in bullet-point form, the major improvements to the manuscript:

- We analyzed replication fork dynamics after additional time periods (specific comment 1). This new data is presented as a supplementary Fig. 1a, 2b and 4. These new results have been also indicated in the main body of the manuscript.
- Chk1 activation has been addressed in response to reviewer 1's comment 1 (see above).
- We performed additional experiments to address the reviewer's query regarding FANCM's role in HU mediated replication arrest.
  The new data (attached to this letter) show that the HU treatment induced a similar response to CPT in FANCM-deficient cells, although overall the effect was somewhat milder even with high doses of HU (see Fig. A attached to this letter). Since the mode by which HU destabilizes replication forks is less well understood, and we do not feel that the lack of these data in our original manuscript weakens our final conclusions, we opted for excluding the new figures in the revised version. However, to highlight the fact that it is very likely that FANCM function may be required to deal with various types of replicative stress we indicate these findings in the main body of the manuscript.

Specific Comment 1: *Fig. 1. The authors show that FANCM-ko cells have more phosphorylated H2AX than wt and attribute such elevated DNA damage to increased collapse of replication forks under unperturbed replication. This might be correct in theory, however, it is quite surprising that DNA fiber analysis does not detect an increased fraction of stalled forks in FANCM-ko cells. One could envisage that H2AX is not labeling collapsed forks (i.e. DSBs) but fork stalling or some post-replication repair of gaps by HR. Indeed, analysis of RAD51 foci might not detect enhancement of RAD51-independent recombination. To clarify these points, the rate of elongation should be analyzed at shorter and longer labeling times with CldU and double-labelling experiments should be employed to assess whether H2AX co-localizes with replication sites. Please include reference bar for the DNA fibers images.*

Our reply: We agree with the reviewer comment that phosphorylated H2AX could be associating with other type of DNA lesions apart from the stalled/collapsed forks. In order to address this concern we performed additional experiments as suggested by the reviewer. We examined the rate of elongation during a shorter (5 min) and a longer (20 min) labeling time (new supplementary figure 1a) as well as the co-localization of gH2AX with sites of replication by double labeling technique (new supplementary figure 1b). We believe that the additional data support our original findings and indicate that indeed, the gH2AX foci in FANCM deficient cells are most likely denoting stalled/collapsed forks.

In our opinion the lack of increased fraction of stalled forks in unperturbed cells could be easily explained in the context of our data. As we show in the manuscript the stalling/collapse of replication fork in FANCM-deficient cells triggers the firing of a neighboring dormant origin of replication. Considering the limitations of the fiber technique employed in this study such an event, in the absence of roscovitine, would be scored as an "ongoing" fork; and indeed we do see an increased fraction of "ongoing forks" in FANCM-deficient cells.

We are a bit confused with respect to the "*Rad-51 independent recombination*" comment. Existing data suggest that Rad51 is absolutely essential for all HR related processes in higher organisms i.e. vertebrates.

Specific Comment 2. *Fig. 2b,c, d. The authors say that loss of FANCM determines inability to restart replication from damaged forks but a faster recovery because of enhanced firing from adjacent origin. However, I have some problem with these experiments. First of all, from the images of DNA fibers obtained in FANCM-ko cells, it seems that the incorporation of IdU is less efficient (red tracks are longer in the wt). Is this a labeling defect or actually loss of FANCM results in a reduced ability to overcome CPT-induced fork stalling? To address this issue, the authors should adopt a different experimental scheme of IdU labeling. Second, even though I am pretty convinced by the roscovitine experiments that firing of new origins is used by FANCM-ko cells to complete DNA replication, I think that additional experiments using at least one shorter and one longer CldU labeling time should be included to confirm actual firing from adjacent origin and to*

make comparison with recovery in wt cells. Please, include in the figure legend details about the X-axis of the graphs showed in panel "a".

We agree with the reviewer's comment that the fiber images in Figure 2a could be "misleading" with respect to the fork velocity in FANCM-deficient cells. We apologize for this unfortunate choice of image. This specific image was selected to reflect the fact that FANCM-deficient cells under replicative stress accumulate, to greater extent than their WT counterparts, various "categories" of replication structures. However, in order to prevent such misconceptions and to more accurately convey the nature of our findings we have calculated the "average" tract length in untreated and roscovitine treated WT and FANCM-deficient cells. This new data indicate that on average, the rate of DNA elongation is statistically not different between WT and FANCM-deficient cells (see below, data not included in the manuscript). The old picture has been replaced with a new one more closely reflecting similar rate of DNA elongation between this two cell lines (see Fig. B attached to this letter).

We are not entirely sure what the reviewer meant by the following: "*use of a different experimental scheme of IdU labeling*". As explained above, we calculated an "average" fork velocity after various labeling periods and this parameter seems to be similar in both cell lines.

We agree with the reviewer's comments regarding the roscovitine experiment and as suggested by the reviewer we performed additional experiments with shorter (5 min) and longer (20 min) CIdU labeling. The new data are now included in supplementary Fig. 2b and 4).

Figure 2 panel a – the details regarding the X-axis have now been added to the figure legend.

Specific Comment 3. *Fig. 3. No conclusion can be drawn without data on total CHK1 levels. From the blot, one could equally conclude that CHK1 gets activated early after CPT addition and then is deactivated. In view of the findings presented later on in the manuscript, CHK1 degradation could also occur to relieve checkpoint arrest.*
Fig. 4. The authors should give a rationale for the choice to check SMC1 phosphorylation as additional ATR target. Most importantly, the results about RPA association with chromatin in wt cells are not consistent with ATR-CHK1 activation. The fractionation protocol used by the authors is very selective and could enrich for proteins that strongly associate with chromatin because, at the best of my experience, a lot of chromatin-bound proteins dissociate at 420mM of NaCl. To make firm conclusions on ssDNA formation, RPA binding and so on, I advice the authors to repeat experiments analyzing also the 420mM fractions. The same applies to TopBP1 chromatin association.
I will not emphasize the deficient ATR activity in FANCM-ko cells because this point could be demonstrated only performing kinase assays using immunopurified ATR. One minor comment: it is quite expected that levels of DSBs are the same in the two genotypes since DSBs form by collision with the replisome. Any difference should stand in the repair of those DSBs.

Our reply:
We agree with the reviewer that the lack of the total Chk1 makes this data difficult for interpretation and apologize for this omission in our original manuscript. This has been now amended (for details see the reply to the reviewer's 1 comment 1).

Fig. 4. The authors should give a rationale for the choice to check SMC1 phosphorylation as additional ATR target.
We have revised that specific part of the manuscript to more clearly connect/reflect the logical flow of the experiments described. Briefly, the reason for checking the Smc1 phosphorylation was that the ablation of claspin selectively abrogates phosphorylation of Chk1 with no effect on other ATR targets (Liu et al, 2006). Therefore, checking these other targets would allow us to assign (position) FANCM in the pathway for Chk1 activation downstream or upstream of claspin. The prediction was that if FANCM functioned upstream of claspin, phosphorylation of Smc1 in a FANCM-deficient cell line should be impaired; and this is indeed what we see (Fig. 3a).

Most importantly, the results about RPA association with chromatin in wt cells are not consistent with ATR-CHK1 activation. The fractionation protocol used by the authors is very selective and could enrich for proteins that strongly associate with chromatin because, at the best of my

experience, a lot of chromatin-bound proteins dissociate at 420mM of NaCl. To make firm conclusions on ssDNA formation, RPA binding and so on, I advice the authors to repeat experiments analyzing also the 420mM fractions. The same applies to TopBP1 chromatin association.
We apologize for the confusion on this point stemming from the omission of some important experimental details regarding our sub-fractionation protocol. In fact, the final salt concentration was 280mM (one part of the hypotonic buffer mixed with two parts of the nuclear extraction buffer - as described in Guo R, Xu D, Wang W, Methods. 2009 May;48(1):72-9). We have now amended this in the materials and methods section. Since RPA association with chromatin is important in testing our hypothesis we repeated this experiment with a new anti-RPA antibody (RPA1) with significantly better cross-reactivity with chicken RPA. The new data confirm our initial observation and is now presented in the new Figure 4b.
I will not emphasize the deficient ATR activity in FANCM-ko cells because this point could be demonstrated only performing kinase assays using immunopurified ATR.
We agree with the reviewer's comment and have modified the main body of the manuscript accordingly.

Fig. 5. It is not possible to affirm that MCM2 is phosphorylated from the blot presented in the panel "a".

Our reply: We apologize for the unconvincing blot presented in figure 5a which we have replaced with an improved one. In addition, we have screened panel of phospho-specyfic antibodies recognizing the equivalent of Xenopus Mcm2 serine 92 in chicken Mcm2 (serine 95). We have repeated the experiment in question using the new antibody. The analysis was done in the presence and absence of caffeine (Reviewer 3 specific comment 1, see below) - in order to strengthen our argument that this specific site on Mcm2 is a target for ATR. To reflect this new data we replaced old Figure 5A with new Figure 5b).

REVIEWER 3

General Comments:

I think this is a nice and original piece of work. The data are logically presented and the experiments are well performed. Most of the data make sense with previous results from other groups. There are few discrepancies that should be addressed, in particular regarding the role of FANCM in ATR activation, before publication is granted.

Our reply: We appreciate the positive comments of this reviewer.

Specific Comment 1: *The authors claim that ATR cannot be activated in the absence of FANCM. In support of this they show that there is no Chk1 phosphorylation in Fig 3a post CPT. However, they show in Fig 5a that MCM is phosphorylated and they speculate that this event is ATR dependent. I am a bit puzzled by the way the data have been presented. Is ATR responsible for direct MCM phosphorylation in these conditions? The authors could address this point by performing the same experiment of Fig 3a in the presence of Caffeine. If the phosphorylation disappears they might speculate that ATR is directly responsible for MCM phosphorylation in FANCM depleted cells.*

Our reply: We are grateful to the reviewer for suggesting an experiment that would help to clarify our hypothesis. Analysis of Mcm2 phosphorylation in the presence and absence of caffeine shows that indeed the phosphorylation of Mcm2 at serine 95 is caffeine-sensitive and as such points to a role of ATR in this process (new Fig. 5b)

Specific Comment 2: *They authors should better discuss the role of FANCM in promoting Chk1 and Smc phosphorylation. From the data presented in which MCM phosphorylation appears to be intact it seems that FANCM is not promoting the activation of ATR but is involved in substrate selection. It is possible that FANCM is holding the fork structure in a permissive state for Chk1 recruitment and phosphorylation. Consistent with this residual TopBP1 binding present in FANCM -/- cells (Fig 4c) would be sufficient to promote local activation of ATR but to enough to ensure Chk1 activation.*

Our reply: We accept that by modulating TopBP1 association with chromatin, FANCM could be involved in ATR substrate selection, albeit indirectly, by controlling the switch between local and global activation of ATR. To reflect this, we have modified the discussion accordingly.

Specific Comment 3: *What is the impact of Plk inhibitors on survival of FANCM -/- cells in unperturbed and damaged conditions? This is an important point to address the physiological significance of these findings.*
Our reply: We agree with the reviewer's comment and are grateful for suggesting this important experiment that would strengthen our original hypothesis. If correct, our hypothesis would predict that inhibiting PLK1 in FANCM-deficient cells would increase their sensitivity to CPT; and indeed this is what we see with regard to survival of FANCM mutant cells in the presence of both PLK1 inhibitors (supplementary Fig. 6).

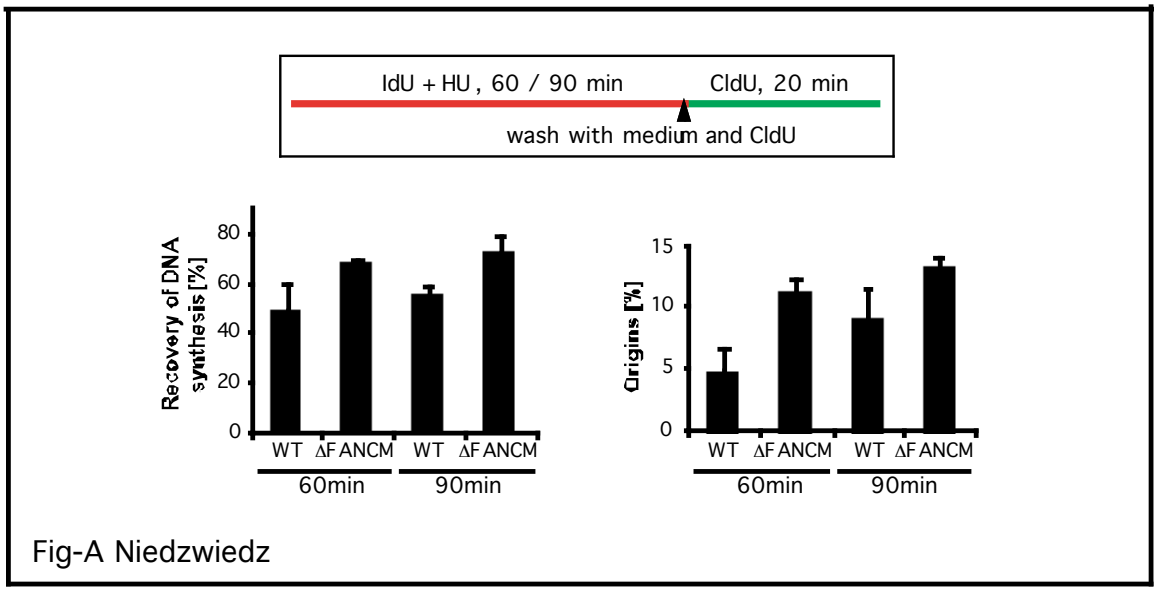

Fig. A. Recovery of DNA synthesis and origin firing in response to hydroxy urea (HU) treatment in WT and DFANCM cells.
Top - Overview of the fiber labeling procedure, below – effect of treating WT and DFANCM cells with 5mM HU for the indicated times on replication fork activity and origin firing.

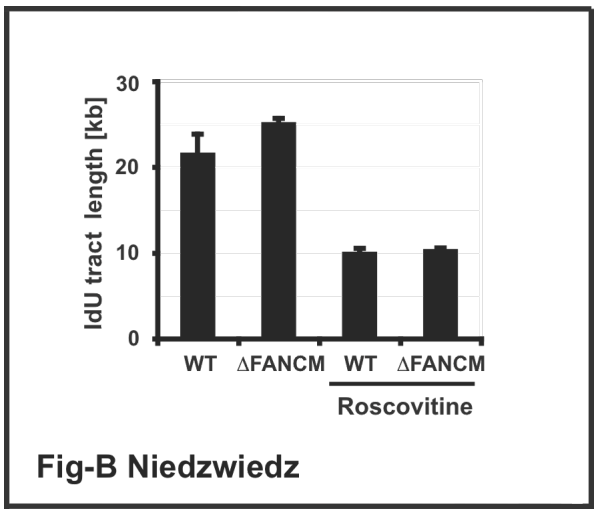

Fig. B. Average IdU tract length in the presence of CPT with or without roscovitine.

WT and DFANCM cells were labeled with 25 mM IdU and simultaneously treated with 2.5 mM CPT with or without roscovitine for 90 min. The resulting tracts were measured and the average was calculated.

2nd Editorial Decision 16 November 2009

Your revised manuscript has been reviewed once more by one of the original referees, who is satisfied with the changes and recommends publication in the EMBO Journal. The manuscript is accepted, however, s/he suggests changes to the discussion to perhaps make a more direct link between checkpoint and FANC function, I leave this to your discretion.

If you decide to make any changes, when you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review. 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 reading the revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORT:

Referee #2 (Remarks to the Author):

"ATR activation and replication fork restart are defective in FANCM-deficient cells" by Rebekka Schwab and colleagues.

I appreciated very much the effort made by the authors to improve their ms. The authors have done a good job of responding to my comments and I feel that the ms is now suitable for publication in the EMBO J.
I have just a very minor comment, that does not preclude a positive opinion about publication.
I would only suggest the authors to revise a little the discussion. At the present, I found it a little bit dispersive. I think the it could benefit from a reduction in lenght and a direct link between checkpoint and FANC function.

2nd Revision - Authors' Response 20 November 2009

Thank you very much for accepting our paper for publication in EMBO Journal.

After careful consideration and conversations with our colleagues we feel that the discussion, in its current form, concisely conveys our conclusions and the implications arising from our work.

