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And-1 coordinates with Claspin for efficient Chk1 activation in response to replication stress

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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.)

Editors: Hartmut Vodermaier

1st Editorial Decision

25 February 2014

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees, whose comments are copied below. As you will see, the referees all appreciate the potential importance of your new findings on Chk1 activation in the ATR pathway, and the role of And-1 in this process. At the same time, they however raise a number of substantial concerns with the experimental evidence, and remain unconvinced that the main conclusions of the study are sufficiently supported by the present set of data. In particular, they criticize the absence of *in vivo* confirmation of the proposed Claspin targeting function of And-1. Another key issue is that the possible role of indirect And-1 depletion effects on S-phase progression cannot be ruled out based on the available evidence. Finally, the reviewers also point out that more decisive analyses on the role And-1 phosphorylation would be required to provide better understanding of the underlying mechanisms.

In light of these significant conceptual problems - which come on top of several specific major concerns that I will not reiterate here - we unfortunately have to conclude that we will not be able to invite and commit to a revision of this study at the present stage, despite the potential interest of these results. As you hopefully understand, we receive a high number of submissions and can therefore only move forward with those few that are met with heightened enthusiasm from at least a majority of referees already upon initial review, and that appear to be sufficiently close to becoming publishable during a limited period of revision.

Should future work allow you to decisively rule out/clarify the key caveats and to extend the mechanistic and in vivo functional analyses as requested by the referees (as well as to address the various more specific points), we would nevertheless remain open to looking once more at this study; which would in this case however have to be treated as a new submission rather than a revision, also with regard to the novelty and advance at the time of resubmission, and only be sent back to the referees if we felt their main concerns had been adequately addressed.

I am sorry that we cannot be more positive at the present stage, but in any case hope that you will find our referees' constructive comments helpful.

Referee #1:

In this paper the authors report that And-1 is an important factor for activation of ATR dependent DNA damage response.

The study starts with the identification of new And-1 binding partners involved in the checkpoint response such as Claspin, RPA subunits and the proteins of the replication pausing complex Timless, Tipin and Claspin. They report that knock down of And-1 expression in HCT116 cells reduces the level of Chk1 phosphorylation in whole cell extracts after UV or HU treatment compared to untreated cells. This observation was rescued using a stable cell line containing And-1 cDNA resistant to siRNA. As far as the mechanism of action is concerned the authors propose that ATR dependent phosphorylation of T826 of And-1 is critical for Claspin interaction with ssDNA leading to Chk1 activation.

Unfortunately, the manuscript lacks some important control experiments. As such, I believe that the manuscript is not ready for publication.

Comments:

- In a previous publication using the same cell lines, the authors have demonstrated that And-1 knockdown leads to massive decrease in S-phase cells population with BrdU incorporation being as follows: 51% in the control and 10% in AND-1 siRNA treated cells, Zhu W. et al., 2007. More recently the authors showed that silencing And1 leads to an important defect in Mcm loading during G1 which is associated with decrease in fork density (Li, Y. et al., 2012).

Considering that only 10% of HCT116 cells incorporate BrdU after transfection with And-1 siRNA, HU dependent activation of Chk1 phosphorylation might not be significant and thus the conclusions of the paper misinterpreted. It would be entirely expected to get lower activation of Chk1 in cells that do not replicate. The authors now discuss this point showing a FACS profile (for which I did not find the Methods) claiming that at 48 hours there is no effects of And-1 depletion. As this is a fundamental control for the interpretation of the results the authors should provide an accurate analysis of the cell cycle profile using BrdU incorporation as in the previous report taking points at 24, 48, 36 and 72 hours to confirm or disprove this point.

- In vitro data support the idea that And-1 is responsible for Claspin binding on ssDNA. This is a key experiment of the paper. However I believe that this finding must be confirmed in vivo for 2 reasons:

a) First, using ssDNA pull down, the authors showed that And1 binds with high affinity ssDNA in comparison to dsDNA. This result is different from the one published by Bermudez VP et colleagues in 2010 where they showed that And-1 has low affinity for ssDNA and high affinity for double to single strand junction. Can the authors explain this discrepancy? Can they provide in vivo evidence that And-1 directly bind ssDNA?

b) Then, Yoshizawa-Sugata and Masai (2009) showed in vivo that And1 does not relocate to chromatin after HU treatment, behavior different to the one observed for RPA. Can the authors better discuss this difference?

-The signals of the Western blot showing Claspin in Flag Chk1 pull down (Fig4B) and Flag And1 pull down (Fig5F) are not clear. These are key experiments of the paper and should be repeated.

Referee #2:

In this manuscript, Hao et al investigate the role of And-1 in response to replication stress. They demonstrate that And-1 interacts with the replication fork proteins, Claspin, Timeless and Tipin, in a manner that is enhanced following HU treatment, and they also show that And-1 is found at sites of DNA damage. Analysis of cellular phenotypes after And-1 knockdown reveals an apparent defect in Chk1 phosphorylation following HU as well as in recovery from replication stress by combing. They show that loss of And-1 decreases the interaction between Claspin and Chk1, and that And-1 phosphorylation by ATR on T826 promotes the interaction of And-1 with claspin, And-1 localization to sites of DNA damage and Chk1 phosphorylation. Finally, in a series of biochemical experiments the authors demonstrate that And-1 interacts with naked ssDNA and promotes the interaction of Claspin with ssDNA via an interaction that requires T826 phosphorylation. These observations tie ATR activation and phosphorylation of And1 to Chk1 activation, and they suggest that And-1 phosphorylation promotes this event by recruiting Claspin and subsequently Chk1 to the fork.

This series of experiments provides new molecular insights into the mechanism of Chk1 activation and the role of And-1 in this process. Given the importance of this pathway and broad interest in the details of ATR-Chk1 signaling, this study has the potential to be of significant interest. Previous studies have hinted at a role for And-1 in Chk1 activation yet were somewhat inconclusive and also lacked mechanistic detail. This study attempts to fill these gaps and significantly extends what is known. Unfortunately, at this point, the study falls short of doing so in a convincing manner. Several of the experiments shown have technical problems that need to be addressed before publication and there are some gaps between what is shown in vitro and in vivo that should be extended/filled.

Major Points/Further Experiments Needed:

- The authors suggest And1 is needed for the Clapsin-Chk1 interaction but is And-1 phosphorylation needed for this interaction? This was not tested and would strengthen the link between And-1 phosphorylation and Chk1 - right now the main experiments are to examine the interaction between And-1 and Claspin.
- The key experiments linking And-1 to Claspin are made in vitro. More in vivo evidence of this would strengthen the conclusions. For example, a test of this model would be to use iPond to determine if Claspin and And1 interactions with the replication fork are affected by the And1 phospho-mutation.
- A major point of this paper is that And-1 phosphorylation helps to direct Chk1 activation and fork recovery. Thus, it is important that the authors test the effect of the And1-phospho mutant on fork recovery after replication stress using stable cell lines expressing this mutant.

Other points:

Figure 1. Can any of the observed interactions be observed with endogenous And1?

Figure 2.

-How long are cells treated with UV before analysis?

-Quantification of the results in Figure 2A, B, C is necessary. Is this all of the cells? or a certain subset? A more representative image (e.g. of multiple cells) should be shown for both full length And-1 and for the truncation mutants as well.

-The authors should validate the antibody used here - does the And1 signal decrease after And-1 knockdown?

Figure 3.

-A more accurate analysis of And-1's effects on S phase should be shown. There does appear to be an effect/reduction in S phase cells from the flow data. The authors should pulse the cells with EdU or BrdU for a short period and determine if the number of S phase cells differs, and if progression has been altered. This could significantly affect Chk1 phosphorylation. In Zhu et al, G&D, it is reported that And1 depletion significantly affects S phase progression after 72 h as these authors discuss, but in Yoshizawa-Sugata et al an effect is seen on S-phase progression after 48 hour knockdown.

-Along these lines, I am having trouble understanding how Pol alpha depletion does not affect S phase progression? The authors should show the extent of Pol alpha depletion in the experiments shown. If the depletion is recent, a dramatic effect on total S phase cells might not yet be evident but effects on S-phase progression could be dramatic.

Figure 4

- Is the increased interaction due to cell cycle synchronization after overnight incubation with HU? What is the effect of HU after 2 hours, the time used for most of the other experiments. Also, is ethidium bromide/DNAse present in these experiments?

- The differences in interactions seen in these experiments may be due to differences in loading. The blot for IPed FLAG-Chk1 is dramatically overexposed but even from this it looks like there may be less Flag-Chk1 in the And1 siRNA + HU lane. This may account for some of the effects observed. Also, it would be helpful to confirm that claspin is what is detected by this antibody (a knockdown of Claspin would be helpful here for specificity) since claspin is highly phosphorylated. Perhaps the species of claspin interacting with chk1 are different in these different conditions. It is also important to show this with endogenous Chk1.

p11 - the text is messed up and several words or even lines are missing

Figure 5a. The pAnd1 blots do not appear to be from the same gel as the IP FLAG blots as the band shapes differ. For this type of experiment I would expect one to blot for pAnd1 then strip and reprobe the blot for FLAG to properly control the experiment/loading.

Fig 5. What is the effect of the ATR inhibitor on localization of wt And1 in cells (e.g. Figure 5) and on the interaction of And1 with Claspin, Timeless and Tipin in the IP experiments (Fig 5)?

In Fig 5E, a more quantitative analysis should be performed

Figure 5G. The expression of the mutant And-1 is not at the same level as the wild-type protein and this may account for the effect on Chk1. This should be done with a higher expression clone and to be certain that the effect is not due to some type of integration issue, ideally with 2 different clones. A functional readout for checkpoint activation (e.g. H3 phosphorylation, G2/M entry) should also be assessed.

Figure 6. Some control to show use of equivalent moles of ss vs dsDNA is needed. The specificity in binding should be confirmed with a gel shift assay. Also, what is the effect of RPA on And-1 interaction with ssDNA. The association of And-1 with naked DNA in a cell could be dramatically altered by RPA.

Figure 6D. Again the blot for And1 loading is overexposed, making it difficult to determine if there are equal amounts of And1 in the experiments - indeed, it seems that there could be more in lane 3. A lighter exposure needs to be used to show that the effect is not due to increased loading. Also, why is there less And1 and claspin binding in the mutant with HU (lane 4) than in the wt with HU (lane 2)? This could be a loading issue as well.

Figure 7. A statistical analysis needs to be done for the combing experiments and the number of fibers analyzed is critical for this type of experiment. See Techer, H, Koundrioukoff, S et al J. Mol Biol (2013) for information on analysis. Also, the fibers shown for siA-1 and siA-2 could represent termination events that increase due to more initiation (which could come from checkpoint activation). To prove that these are defects in fork progression further experiments are required. For example, the authors could:

- Confirm that Inter-Origin distance is the same
- Measure forks asymmetry, another indicator of fork collapse.
- Quantify the CldU incorporation, to be sure there is less CldU incorporation in their mutants.

Referee #3:

In this manuscript, Hao and colleagues address the role of And-1 in the cellular response to replication stress. And-1 is a replisome component that is required for the recruitment of DNA pol alpha to chromatin and for normal DNA replication. The main finding of this paper is that And-1 interacts physically with the checkpoint mediator Claspin and is phosphorylated by ATR on T826 in response to hydroxyurea (HU). Importantly, this phosphorylation promotes the interaction between And-1 and Claspin and is required for the full activation of Chk1. They also show that And-1 single-stranded DNA-binding protein and propose an original model in which And-1 promotes the recruitment of Claspin and Chk1 at stalled replication forks in an ATR-dependent manner. Overall, this is a very nice and complete story that brings new light on the mechanism by which ATR activates Chk1 in response to replication stress. Considering that this mechanism is still unknown almost 20 years after the discovery of ATR, this implication of And-1 represents a significant advance. The manuscript is clear and the experiments are of high quality. However, several important issues need to be addressed:

Major points:

- 1- The authors indicate that And-1 accumulates at "damage sites" (Fig. 2), but they should clarify to what type of "DNA damage" they refer to. In panel 2A, they expose cells to 10 mM HU for 2 hrs, which is not sufficient to induce DNA breaks. In this case, And-1 is probably recruited to paused, undamaged forks. In panel 2D, And-1 is recruited to UV-damaged DNA, but it is not clear whether cells with foci are in S phase or not. In other words, it could be that And-1 is recruited to damaged sites, independently of the replication fork. The authors should address this possibility by labeling cells with BrdU or EdU prior to UV exposure. Along the same line, it is not surprising that the MCM7 signal does not strictly colocalize with damage sites as it binds unreplicated chromatin and does not even colocalize with replication foci. Yet, it would be important to test whether And-1 colocalizes with replication foci in the absence of HU or UV treatment.
- 2- The fact that And-1 binds ssDNA in vitro is interesting, but the fact that it does in vivo is not demonstrated. The authors should tone down this statement in the manuscript. Moreover, since Tipin-RPA interaction is required to stabilize Claspin on RPA-coated ssDNA and to activate Chk1, the authors should ask whether RPA loading on chromatin in HU is affected when And-1 is absent. Is there a modification of RPA-And-1 interaction in cells expressing the And-1 T826 mutant?
- 3- To firmly establish that AND-1 phosphorylation at T826 is important for Claspin-Chk1 interaction, the authors should repeat the experiment shown Fig. 4B in cells expressing the AND-1 mutant T826A.
- 4- The DNA combing experiments are convincing, but the authors need to indicate how many fibers/forks were analyzed. They should also measure the length of IdU and CldU tracks to determine whether the depletion of And-1 affects the speed of replication forks, both in the presence and the absence of HU.
- 5- The discussion is too long and too defensive, especially regarding the results of Yoshizawa et al. This study also showed that Cdc7 contributes to And-1 phosphorylation. This issue should be discussed, if relevant.
- 6- It should be mentioned that Ctf4, the yeast homolog of And-1, interacts with multiple components of the replisome and travels with replication forks in unchallenged growth conditions (Gambus et al (2006) Nat Cell Biol 8, 358).

Minor points :

- 1- P8 : The paragraph on whether And-1 is required for initiation or amplification of Chk1 activation relies on fairly weak data (not exactly the same kinetics in HU and UV, difficulty to discriminate between initiation and amplification with this experimental design). Fig. 3D should move to supplemental data and the paragraph should be shortened, as this is not a very important point of the paper.
- 2- P11: The sentence "... indicating that ATR is the primary kinase that..." is incomplete.
- 3- Fig. 7A : The experimental design is not clear. Indicate that cells were released from HU treatment. In the legend, add the concentration of HU that was used.

Referee #1:

1. Unfortunately, the manuscript lacks some important control experiments. As such, I believe that the manuscript is not ready for publication.

We have conducted and provided all control experiments as requested. See below for detail.

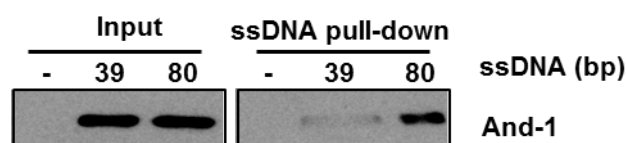
2. In a previous publication using the same cell lines, the authors have demonstrated that And-1 knockdown leads to massive decrease in S-phase cells population with BrdU incorporation being as follows: 51% in the control and 10% in AND-1 siRNA treated cells, Zhu W. et al., 2007. More recently the authors showed that silencing And1 leads to an important defect in Mcm loading during G1 which is associated with decrease in fork density (Li, Y. et al., 2012). Considering that only 10% of HCT116 cells incorporate BrdU after transfection with And-1 siRNA, HU dependent activation of Chk1 phosphorylation might not be significant and thus the conclusions of the paper misinterpreted. It would be entirely expected to get lower activation of Chk1 in cells that do not replicate. The authors now discuss this point showing a FACS profile (for which I did not find the Methods) claiming that at 48 hours there is no effects of And-1 depletion. As this is a fundamental control for the interpretation of the results the authors should provide an accurate analysis of the cell cycle profile using BrdU incorporation as in the previous report taking points at 24, 48, 36 and 72 hours to confirm or disprove this point.

As the reviewer suggested, we have performed an EdU incorporation assay to examine the cell cycle effects of And-1 down-regulation and the data is shown in supplementary Fig S3. Our data show that at 48 hours after And-1 knockdown, there is a slight decrease of S phase cells (about 5%). Thus, we think that Chk1 activation defect at 48 hours after And-1 depletion is not due to the cell cycle effect. In addition, We previously published data showing that at 48 hours after And-1 depletion, Pol alpha protein level is reduced mildly (Fig S2B, Zhu et al., Genes Dev. 2007 21(18):2288). This explains why we did not observe significant effects of a 48-hr And-1 depletion on DNA synthesis and cell cycle progression. In agreement with our conclusion, Yoshizawa-Sugata et al. have demonstrated that at 48 hours after And-1 siRNA transfection, there is no significant alteration in cell proliferation (Fig. 2B, Yoshizawa-Sugata et al., J Biol Chem. 2009 284:20718-28).

3. In vitro data support the idea that And-1 is responsible for Claspin binding on ssDNA. This is a key experiment of the paper. However I believe that this finding must be confirmed in vivo for 2 reasons:

a) First, using ssDNA pull down, the authors showed that And1 binds with high affinity ssDNA in comparison to dsDNA. This result is different from the one published by Bermudez VP et colleagues in 2010 where they showed that And-1 has low affinity for ssDNA and high affinity for double to single strand junction. Can the authors explain this discrepancy?

The paper published by Bermudez VP et al. showed that And-1 binds to ssDNA but the affinity for ssDNA is low. We believe that this discrepancy is due to the length of ssDNA molecule used in the two studies. In our study, we used a biotin labelled 80bp ssDNA whereas in their study a 39bp ssDNA was used. It could be that this 39bp ssDNA is too short for effective protein binding. To support our notion, we performed an experiment using equal molar amount of 80bp or 39bp ssDNA molecules to test the affinity of And-1 to ssDNA. The data below show that And-1 has higher affinity with 80bp ssDNA than 39bp ssDNA.



Can they provide in vivo evidence that And-1 directly bind ssDNA?

The answer is yes. We have performed in vivo experiment to demonstrate that And-1 clearly co-localizes with ssDNA (Fig 6E). In addition, we also conducted iPOND assay as requested by the second reviewer and these data indicate that And-1 accumulates at stalled replication. The iPOND data are included in Figs. 6F&G.

b) Then, Yoshizawa-Sugata and Masai (2009) showed in vivo that And1 does not relocate to chromatin after HU treatment, behavior different to the one observed for RPA. Can the authors better discuss this difference?

Compared to RPA, And-1 is a much more abundant nucleic protein. Due to this fact, we have to pre-extract nuclei to remove non-chromatin associated And-1 in order to observe And-1 foci at damage sites (see our method for detail). Thus, it appears that a small portion of And-1 is recruited to DNA damage site to regulate checkpoint activation, while most RPA proteins are involved in checkpoint activation. Since chromatin association experiments showed increased chromatin association of RPA but not And-1, we think that the best and precise approach to demonstrate the localization of proteins to damage sites is immunostaining of pre-extracted nuclei and other in vivo assays (such as iPOND). Indeed, our data from immunostaining and iPOND analyses clearly shows that And-1 is localized at DNA damage sites (Figs. 2A, B, 5E & 6E-F).

4. The signals of the Western blot showing Claspin in Flag Chk1 pull down (Fig4B) and Flag And1 pull down (Fig5F) are not clear. These are key experiments of the paper and should be repeated.

We have repeated experiments concerning the Western blotting data in Fig. 4B and Fig. 5F. The new data agrees with the old data and is now included in Figure 4B and Figure 5G respectively.

Referee #2:

Major Points/Further Experiments Needed:

1. The authors suggest And1 is needed for the Claspin-Chk1 interaction but is And-1 phosphorylation needed for this interaction? This was not tested and would strengthen the link between And-1 phosphorylation and Chk1 - right now the main experiments are to examine the interaction between And-1 and Claspin.

Thank you for the suggestion. We have now included data (supplementary Fig S4) showing that And-1 phosphorylation is required for the interaction between Claspin and Chk1.

2. The key experiments linking And-1 to Claspin are made in vitro. More in vivo evidence of this would strengthen the conclusions. For example, a test of this model would be to use iPOND to determine if Claspin and And1 interactions with the replication fork are affected by the And1 phospho-mutation.

We respectfully disagree.

In the first submission, we showed that And-1 regulates the interaction between Chk1 and Claspin and phosphorylation of And-1 is required for its association with Claspin in vivo.

Following your suggestion, we have spent a great amount of time and effort to set up iPOND assay to examine the association of Claspin, wild type And-1 and mutant And-1 (T826A) with replication forks. We have now included the iPOND data in Figures. 6F & G. The data clearly shows that And-1 associates with replication forks and that the wild type And-1 but not mutant And-1 (T826A) strongly interacts with stalled replication forks.

We have also spent great efforts to demonstrate that Claspin is a fork-associated protein by iPOND. Our effort failed to demonstrate it. The main reason for the failure is simple - the reverse cross-link could not recover Claspin proteins. This failure had nothing to do with our reagents because we have tried every commercially available Claspin antibody under various conditions using iPOND assay. Although we failed to detect the association of Claspin with replication forks, others have reported that Claspin is a replisome component that associates with replication forks (Gambus et al. 2006, Nat Cell Biol 8, 358)

3. A major point of this paper is that And-1 phosphorylation helps to direct Chk1 activation and fork recovery. Thus, it is important that the authors test the effect of the And1-phospho mutant on fork recovery after replication stress using stable cell lines expressing this mutant.

The major goal of this study is to determine the molecular mechanism by which And-1 regulates ATR-Chk1 pathway via Claspin, which we have done. It is beyond the scope of this study to investigate the detailed mechanism of how And-1 regulates fork stability. Since Scolah et al (Scolah et al., Cell Cycle. 2009 Apr 1; 8(7): 1036–1043.) reported that Claspin is required to maintain fork stability, the simple goal for us was to examine whether And-1 regulates fork recover via the same pathway as Claspin.

Nevertheless, we greatly appreciate this suggestion. Unfortunately, the personnel and facility are no longer available for us to conduct DNA combing assays.

Other points:

4. Figure 1. Can any of the observed interactions be observed with endogenous And1?

We have detected the endogenous And-1-Claspin interaction and the new data are included in Fig S1A.

5. Figure 2.

-How long are cells treated with UV before analysis?

After treated with UV, the cells were incubated in the medium for 2 hours before subjected to immunostaining.

-Quantification of the results in Figure 2A, B, C is necessary. Is this all of the cells? or a certain subset? A more representative image (e.g. of multiple cells) should be shown for both full length And-1 and for the truncation mutants as well.

We have re-conducted these experiments and the new data with quantification are now included in Figure 2.

-The authors should validate the antibody used here - does the And1 signal decrease after And-1 knockdown?

We validated our And-1 antibody in a previous publication (Fig 4C, Jaramillo-Lambert, et al., J Biol Chem. 2013; 288(3): 1480–1488.).

6. Figure 3.

-A more accurate analysis of And-1's effects on S phase should be shown. There does appear to be an effect/reduction in S phase cells from the flow data. The authors should pulse the cells with EdU or BrdU for a short period and determine if the number of S phase cells differs, and if progression has been altered. This could significantly affect Chk1 phosphorylation. In Zhu et al, G&D, it is reported that And1 depletion significantly affects S phase progression after 72 h as these authors discuss, but in Yoshizawa-Sugata et al an effect is seen on S-phase progression after 48 hour knockdown.

The concern on the cell cycle effect was also raised by the first reviewer. We have conducted experiments as requested. See response to reviewer 1.

In the paper by Yoshizawa-Sugata et al., the effects on cell cycle and DNA replication by And-1 depletion are conducted in cells 66 hours after And-1 siRNA transfection (See Fig. S1, Yoshizawa-Sugata et al., J Biol Chem. 2009 284:20718-28). Actually, Yoshizawa-Sugata et al. showed that at 48 hours after siRNA transfection, there are no significant cell proliferation effects (Fig. 2B, Yoshizawa-Sugata et al., J Biol Chem. 2009 284:20718-28). These data support our conclusion that 48 hours after And-1 siRNA transfection, there is no significant cell cycle and DNA replication effects.

7. Along these lines, I am having trouble understanding how Pol alpha depletion does not affect S phase progression? The authors should show the extent of Pol alpha depletion in the experiments shown. If the depletion is recent, a dramatic effect on total S phase cells might not yet be evident but effects on S-phase progression could be dramatic.

Yoshizawa-Sugata et al showed that at 48 hours after And-1 siRNA transfection, there was no significant cell proliferation effects (Fig. 2B, Yoshizawa-Sugata et al., J Biol Chem. 2009 284(31):20718-28). We previously published data showing that at 48 hours after And-1 depletion, Pol alpha proteins is reduced mildly (Figure S 2B, Zhu et al., Genes Dev. 2007;21(18):2288-99.). This explains why we did not observe significant effects on DNA synthesis and cell cycle progression at the 48 hours after And-1 depletion.

8. Figure 4

- Is the increased interaction due to cell cycle synchronization after overnight incubation with HU? What is the effect of HU after 2 hours, the time used for most of the other experiments. Also, is ethidium bromide/DNAse present in these experiments?

The time used for all other HU treatment experiments are two hours except experiments shown in Figs. 4A and 5G. We have examined the interactions after 2 hrs treatment and found that there are no significant increases. The key point we want to address from this experiment is that the interaction of And-1 (T826A) with Claspin are not increased under the same treatment and therefore And-1 phosphorylation is critical for Claspin-And-1 interaction.

We used ethidium bromide/DNAse in our IP experiments.

The differences in interactions seen in these experiments may be due to differences in loading. The blot for IPed FLAG-Chk1 is dramatically overexposed but even from this it looks like there may be less Flag-Chk1 in the And1 siRNA + HU lane. This may account for some of the effects observed.

We have re-done the experiment concerning the data in Figure 4B. The new data are now included in Figure 4B.

Also, it would be helpful to confirm that claspin is what is detected by this antibody (a knockdown of Claspin would be helpful here for specificity) since claspin his highly phosphorylated. Perhaps the species of claspin interacting with chk1 are different in these different conditions. It is also important to show this with endogenous Chk1.

We have confirmed the specificity of the Claspin antibody in an experiment as suggested. The new data is included in supplementary Figure S1B. We have examined the interaction of endogenous Chk1-Claspin using three commercial Claspin antibodies but failed. To our knowledge, only one lab has reported the interaction between endogenous Chk1 and Claspin proteins using a customized Claspin antibody. Unfortunately this lab no longer has this customized Claspin antibody.

9. p11 - *the text is messed up and several words or even lines are missing*

The mistakes have been corrected.

10. Figure 5a. The pAnd1 blots do not appear to be from the same gel as the IP FLAG blots as the band shapes differ. For this type of experiment I would expect one to blot for pAnd1 then strip and reprobe the blot for FLAG to properly control the experiment/loading.

We have re-done these experiments and new data are included in Figure 5A.

11. Fig 5. What is the effect of the ATR inhibitor on localization of wt And1 in cells (e.g. Figure 5) and on the interaction of And1 with Claspin, Timeless and Tipin in the IP experiments (Fig 5)?

We have included new data using an ATR inhibitor in supplementary Figure S4B and C. Our data indicate that the localization of And-1 to damage sites is reduced in cells treated with ATR inhibitor. And-1 positive cells were significantly reduced in caffeine treated cells.

We tried both Caffeine and ATR specific inhibitor VE-821 to do the co-IP experiments described in Figure 5G. But both drugs reduces the exogenous And-1 expression in the cells. So presently we could not provide meaningful data to address this matter.

12. In Fig 5E, a more quantitative analysis should be performed

We have quantified the data as requested.

13. Figure 5G. The expression of the mutant And-1 is not at the same level as the wild-type protein and this may account for the effect on Chk1. This should be done with a higher expression clone and to be certain that the effect is not due to some type of integration issue, ideally with 2 different clones. A functional readout for checkpoint activation (e.g. H3 phosphorylation, G2/M entry) should also be assessed.

It is true that in Figure 5E, And-1(WT) expression is a little more than mutant And-1 (less than two-fold). Our data indicate that mutant And-1 expression is the same as And-1 in control siRNA treated cells but failed restore p-Chk1. In Fig. 3C, we have presented another rescue experiment, in which exogenous And-1 expression is at the same level as endogenous And-1 in siRNA control treated cells and could restore p-Chk1. Taking these results together we conclude that And-1(WT) but not mutant can restore p-Chk1.

The major point of this work is to investigate how And-1 regulates DNA replication checkpoint in S phase, for which we have provided a large amount of data. Examination of H3 phosphorylation and G2-M phase is about G2/M checkpoint, which is unrelated to our current investigation and is beyond the scope of this study.

13. Figure 6. Some control to show use of equivalent moles of ss vs dsDNA is needed. The specificity in binding should be confirmed with a gel shift assay. Also, what is the effect of RPA on And-1 interaction with ssDNA. The association of And-1 with naked DNA in a cell could be dramatically altered by RPA.

To ensure we have equal amount of ssDNA or dsDNA, we incubated all the beads with DNA first followed by extensive wash. After washing, we then divided beads equally to each sample. This is a standard approach that has been used widely in the field (Unsal-Kaçmaz et al. Mol Cell Biol. 2004 Feb;24(3):1292-300.; Liu et al. Nat Struct Mol Biol. 2010 Oct; 17(10): 1260–1262.; Shiotani et al. Mol Cell. 2009 Mar 13;33(5):547-58.). To satisfy the reviewer's concern we did run a polyacrylamide gel to show the DNA bands. Because there is very little amount of DNA in each sample, the actual ssDNA band is invisible.

We have conducted experiments to examine whether RPA regulates And-1 interacts with ssDNA. As shown in supplementary Figure. S5D and E, RPA and And-1 bind to ssDNA independently.

14. Figure 6D. Again the blot for And1 loading is overexposed, making it difficult to determine if there are equal amounts of And1 in the experiments - indeed, it seems that there could be more in lane 3. A lighter exposure needs to be used to show that the effect is not due to increased loading. Also, why is there less And1 and claspin binding in the mutant with HU (lane 4) than in the wt with HU (lane 2)? This could be a loading issue as well.

We have re-done these experiments and new data are consistent with old data. The results are shown in Figure 6D.

15. Figure 7. A statistical analysis needs to be done for the combing experiments and the number of fibers analyzed is critical for this type of experiment. See Techer, H, Koundrioukoff, S et al J. Mol

Biol (2013) for information on analysis. Also, the fibers shown for siA-1 and siA-2 could represent termination events that increase due to more initiation (which could come from checkpoint activation). To prove that these are defects in fork progression further experiments are required. For example, the authors could:

- Confirm that Inter-Origin distance is the same
- Measure forks asymmetry, another indicator of fork collapse.
- Quantify the CldU incorporation, to be sure there is less CldU incorporation in their mutants.

We have revised these data by adding the number of fibers.

The major goal of this study is to determine the molecular mechanism by which And-1 regulates ATR-Chk1 pathway via Claspin, which we have done. We did not aim to investigate the detailed mechanism of how And-1 regulates fork stability. Since Scora et al (Scora et al., Cell Cycle. 2009 Apr 1; 8(7): 1036–1043.) reported that Claspin is required to maintain fork stability, the simple goal for us was to examine whether And-1 regulates fork recover via the same pathway as Claspin.

Nevertheless, we greatly appreciate this suggestion. Unfortunately, the personnel and facility are no longer available for us to conduct DNA combing assays.

Referee #3:

Major points:

1- The authors indicate that And-1 accumulates at "damage sites" (Fig. 2), but they should clarify to what type of "DNA damage" they refer to. In panel 2A, they expose cells to 10 mM HU for 2 hrs, which is not sufficient to induce DNA breaks. In this case, And-1 is probably recruited to paused, undamaged forks. In panel 2D, And-1 is recruited to UV-damaged DNA, but it is not clear whether cells with foci are in S phase or not. In other words, it could be that And-1 is recruited to damaged sites, independently of the replication fork. The authors should address this possibility by labeling cells with BrdU or EdU prior to UV exposure. Along the same line, it is not surprising that the MCM7 signal does not strictly colocalize with damage sites as it binds unreplicated chromatin and does not even colocalize with replication foci. Yet, it would be important to test whether And-1 colocalizes with replication foci in the absence of HU or UV treatment.

We appreciate your suggestion. We have conducted experiments in cells labeled with BrdU prior to UV exposure. Our data indicate that And-1 co-localizes with ssDNA in vivo (Figure 6E). To test whether And-1 co-localizes with replication forks in the absence of HU or UV, we have conducted iPOND experiments. Our new data indicate that And-1 is associated with replication forks and accumulated at stalled forks (Figure 6F and G).

2- The fact that And-1 binds ssDNA in vitro is interesting, but the fact that it does in vivo is not demonstrated. The authors should tone down this statement in the manuscript.

Our new data indicate that And-1 co-localizes with ssDNA in vivo (Figure 6E).

Moreover, since Tipin-RPA interaction is required to stabilize Claspin on RPA-coated ssDNA and to activate Chk1, the authors should ask whether RPA loading on chromatin is HU is affected when And-1 is absent. Is there a modification of RPA-And-1 interaction in cells expressing the And-1 T826 mutant?

We examined the chromatin associated RPA in And-1 depleted cells (supplementary Figure S5). We found the amount of chromatin-associated RPA was mildly reduced after HU treatment while the total amount of RPA did not change (supplementary Figure S5B and C). We also found RPA-And-1(T826A) interaction is the same as the RPA-And-1(WT) when HU is applied (Figure 5G).

We also found that the association of RPA and And-1 with ssDNA is not interdependent in vitro (supplementary Figure 5D and E).

3- To firmly establish that AND-1 phosphorylation at T826 is important for Claspin-Chk1 interaction, the authors should repeat the experiment shown Fig. 4B in cells expressing the AND-1 mutant T826A.

We have repeated these experiments and the new data is included in Figure 4B.

4- The DNA combing experiments are convincing, but the authors need to indicate how many fibers/forks were analyzed. They should also measure the length of IdU and CldU tracks to determine whether the depletion of And-1 affects the speed of replication forks, both in the presence and the absence of HU.

See response 15 to reviewer 2.

5- The discussion is too long and too defensive, especially regarding the results of Yoshizawa et al. This study also showed that Cdc7 contributes to And-1 phosphorylation. This issue should be discussed, if relevant.

We have revised our discussion. Regarding Cdc7 contribution, we have never observed an upshift And-1 band.

6- It should be mentioned that Ctf4, the yeast homolog of And-1, interacts with multiple components of the replisome and travels with replication forks in unchallenged growth conditions (Gambus et al (2006) Nat Cell Biol 8, 358).

Thanks for suggestions. We have revised MS regarding this concern.

Minor points :

1- P8 : The paragraph on whether And-1 is required for initiation or amplification of Chk1 activation relies on fairly weak data (not exactly the same kinetics in HU and UV, difficulty to discriminate between initiation and amplification with this experimental design). Fig. 3D should move to supplemental data and the paragraph should be shortened, as this is not a very important point of the paper.

We agree with you and have revised this part.

2- P11: The sentence "... indicating that ATR is the primary kinase that..." is incomplete.

Thanks for the advice. We have revised it accordingly.

3- Fig. 7A : The experimental design is not clear. Indicate that cells were released from HU treatment. In the legend, add the concentration of HU that was used.

Thanks for the suggestions. We have revised these parts.

Editorial Decision

18 March 2015

Thank you for submitting a new version of your manuscript on Chk1 regulation by And-1 for our editorial consideration. It has now been reviewed once more by the three original referees, and I am pleased to inform you that they all consider the study significantly improved and most of the major concerns adequately addressed. Pending further revision of a number of specific issues, we should therefore be able to proceed further with publication in The EMBO Journal.

I am therefore returning the manuscript to you once more for a final round of (minor) revision, inviting you to address the important remaining points raised by referees 2 and 3. In particular, I feel it will be important to address point 1 of referee 2 regarding the need for non-label chasing in And-1 iPOND assays, in order to strengthen the important conclusions on And-1 presence at replication forks.

When re-revising the manuscript, please also consider the following editorial points:

- Please make sure to also introduce the reader to the And-1 homolog Ctf4 and briefly discuss its known functions in relation to the present results.
- Please submit a completed 'author checklist' at the time of resubmission, after having considered the points raised in the checklist.
- Please upload individual figure files in sufficient quality/resolution for eventual production; the current file contains too many compression artifacts for proper assessment.
- Furthermore, in order to make the primary data behind the various blot/gel panels more accessible and more directly represented, I would kindly ask you to include figure source data for the gels, blots and autoradiographs in both the main and the supplementary figures. We would ask for a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed scans of all gel/blot panels used in the respective figures. These should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files can be uploaded upon resubmission selecting "Figure Source Data" as object type, and they would be linked as such to the respective figures in the online publication of your article.
- Finally, please provide us (in your resubmission cover letter) with 2-5 short one-sentence 'bullet points', containing brief factual statements that summarize key aspects of the paper - they will accompany the online version of the article as part of a 'synopsis'. Please see the latest research articles on our website (emboj.embopress.org) for examples - I am happy to offer further guidance on this if necessary.

I hope you will be able to make the necessary further revisions and resubmit a final version of the manuscript as early as possible. Should you have any further questions in this regard, please do not hesitate to get back to me.

Referee #1:

The authors have satisfactorily addressed my critiques.

Referee #2:

The authors have addressed my major concerns and added several new experiments that strengthen the conclusions of this manuscript. There remain some relatively minor technical issues that should be addressed if possible.

1. To show something is at the replication fork by iPond, the samples should be chased with unlabeled media. Right now they demonstrate that And-1 is on chromatin. To place And-1 at the fork, they need to include this chase. It is likely that this is the case with And-1, but not definitive so this should at a minimum be acknowledged.
2. Re the data in Figure 6G and the effect of T862A on binding forks, please clarify if quantification is based only on this experiment or on this plus replicates. The result as shown is not convincing.
3. The author should include quantification of data shown in Figure 6E.
4. Point 7 of review #2 was why Pol alpha did not affect S phase progression and Chk1 phosphorylation, not what the effect of And1 knockdown is. This is a minor point however.
5. Several supplemental figures are not mentioned in the text. These should be referred to as the reason some are included is not clear. For example, why are Fig S5B and C included. They seem to show that And1 affects the stability of RPA70 binding to chromatin.

6. The model for the experiments shown in Fig7A does not match the description provided. Shouldn't the HU treatment be between the Idu and CldU pulses.

Referee #3:

The authors have provided many new experiments and have properly addressed most - if not all - the issues raised by the referees. In my opinion, the manuscript is now suitable for publication in EMBO Journal. However, the following minor points must be addressed prior to publication.

Page 7: "We observed formation of HU induced And-1 foci in a portion of HCT116 cells that co-localized with gamma-H2AX and RPA70 foci." Indicate the % of cells that present colocalised foci.

Page 9: ATR and not ART

Fig.6E: Indicate the fraction of cells showing colocalisation of the signals

Fig.7A: The experimental scheme is not clear: indicate that CldU is added after HU removal

Page 16: "However, depletion of And-1 increased the fraction of forks that failed to recover from HU treatment by 3~4 fold (Fig 7C)". Compared to Ctrl cells, the difference is only 2-3 fold increase in HU.

Fig.S4A: showing the implication of and-1 phosphorylation in chk1-clsp interaction is not so convincing. But it's not the main message in the paper and it's was indirectly suggested by others results. SO it's OK.

Revision - authors' response

30 April 2015

Referee #2:

1. To show something is at the replication fork by iPond, the samples should be chased with unlabeled media. Right now they demonstrate that And-1 is on chromatin. To place And-1 at the fork, they need to include this chase. It is likely that this is the case with And-1, but not definitive so this should at a minimum be acknowledged.

As requested, we have chased the cells in regular medium after EdU label. We found that more And-1 protein specifically accumulated on replication forks. These new data are included in Figure 6G.

2. Re the data in Figure 6G and the effect of T862A on binding forks, please clarify if quantification is based only on this experiment or on this plus replicates. The result as shown is not convincing.

We have quantified the data from three independent experiments and presented the quantification of fork associated And-1 protein intensity in Figure 6H. This information is now in the legend to Fig. 6H.

3. The author should include quantification of data shown in Figure 6E.

We have quantified the data. The quantification is described in figure legend of Figure 6E.

4. Point 7 of review #2 was why Pol alpha did not affect S phase progression and Chk1 phosphorylation, not what the effect of And1 knockdown is. This is a minor point however.

Our data indicate that pol alpha does not affect Chk1 phosphorylation (Fig. 3D). To our knowledge, there is no study indicating that Pol alpha affects Chk1 activation. We did not examine the cell cycle effects of pol alpha depleted cells because it is beyond the scope of this study.

5. Several supplemental figures are not mentioned in the text. These should be referred to as the reason some are included is not clear. For example, why are Fig S5B and C included.

We have revised the text part based on the scope of this research project.

6. The model for the experiments shown in Fig7A does not match the description provided. Shouldn't the HU treatment be between the Idu and CldU pulses.

Thanks for the comment. We have revised this part to avoid confusion.

Referee #3:

The authors have provided many new experiments and have properly addressed most - if not all - the issues raised by the referees. In my opinion, the manuscript is now suitable for publication in EMBO Journal. However, the following minor points must be addressed prior to publication.

Page 7: "We observed formation of HU induced And-1 foci in a portion of HCT116 cells that co-localized with gamma-H2AX and RPA70 foci." Indicate the % of cells that present colocalised foci.

We have added the data in the figure legend (Fig. S4).

Page 9: ATR and not ART

The misspelling is corrected.

Fig.6E: Indicate the fraction of cells showing colocalisation of the signals

The percentage of cells with both BrdU and And-1 staining is described in the figure legend.

Fig.7A: The experimental scheme is not clear: indicate that CldU is added after HU removal

Done.

Page 16: "However, depletion of And-1 increased the fraction of forks that failed to recover from HU treatment by 3~4 fold (Fig 7C)". Compared to Ctrl cells, the difference is only 2-3 fold increase in HU.

We have corrected the description.