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ATM/Wip1 activities at chromatin control Plk1 re-activation to determine G2 checkpoint duration

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Additional communication (editor) 20 December 2016

Thank you again for submitting your manuscript on ATM/Wip1 chromatin roles and Plk1 reactivation after DNA damage checkpoint arrest. We have now received reports from three expert referees, which I am enclosing below for your information. As you will see, all reviewers consider the study potentially interesting, but they do raise a number of specific points, which I would like to pre-discuss with you to see how you might be able to respond to them and address them, and whether this could be achieved within a reasonable revision time frame.

As far as my reading of the reports from the cell-biological referees 1 and 2 goes, their concerns appear to be largely with the validation of the tools and the conclusiveness of the some of the data, as well as with general presentation/interpretation issues, but they do not seem to request conceptually further-reaching mechanistic investigations; I would be interested in hearing how you might answer these points experimentally and/or via other clarifications and discussions.

Referee 3 was asked to specifically look at the mathematical modeling of checkpoint response and cell cycle progression, and unfortunately considers the present modeling insufficient to support major conclusions, so it would be important to hear whether you would be in a position to improve the modeling along the lines suggested, or to validate its predictions experimentally as also echoed by referee 1.

I am therefore giving you an opportunity to consider the enclosed reports and discuss them with your coworkers, and to contact me with with a point-by-point proposal for addressing the referee

comments and an estimate how long this would take. Based on this, we could then discuss the possibility of a revision and its exact requirements ahead of taking a definitive decision here. I would appreciate if you could send us such a response at your earliest convenience, ideally (in light of the upcoming holidays) already by Wednesday evening.

REFEREE REPORTS

Referee #1

(Report for Author)
This paper uses a Fret based sensor to monitor free nuclear or chromatin bound ATM activity to assess the regulation of the G2/M checkpoint after DSB induction, with the focus being on Plk1 activity. It is shown that Plk1 activity after NCS decreases and then returns and that loss of the chromatin bound ATM sensor signal (but not free nuclear signal) correlates with the increase in Plk1 activity, which precedes mitotic entry. It is concluded that a genome wide chromatin bound ATM signal regulates the initial decrease in Plk1 activity and its subsequent return. The ATM signal is also influenced by Wip1, leading to the model that a chromatin bound ATM/Wip1 regulated signal determines the initial duration of checkpoint arrest. However, ATR also influences the rate of return of Plk1 activity ie its influence arises at later times after DSB induction (achieved using NCS). At face value this is consistent with the notion that DSBs undergo resection in G2 phase leading to a switch from ATM to ATR activity. Interestingly, however, mitotic entry arises even in the presence of ATR inhibitors, suggesting that it cannot fully prevent entry. Previous studies have shown that mitotic entry occurs prior to the completion of DSB repair and this is consolidated here showing that when Plk1 activity returns and mitotic entry occurs, gH2AX foci, active ATM at foci and non-chromatin bound ATM activity persist. Thus, the model proposed is that a chromatin bound ATM/Wip-counteracted signal causes an initial decrease in Plk1 activity, which is then sustained by ATR activity. This is distinct to a model whereby ATM (and later ATR) activity at DSBs regulates the duration of checkpoint arrest. It is suggested that this allows ATM's function in DSB repair to be sustained even though mitotic re-entry can occur. Kap1 is chromatin bound and phosphorylated by ATM and the duration of its phosphorylation correlates with the return of Plk1 activity. This represents a complex but interesting paper and, importantly, provides evidence for a novel mechanism for the regulation of mitotic entry after DSB induction. Although there are aspects that appear unclear (discussed below), the notion that the mitotic entry signal is regulated by a pan nuclear chromatin bound ATM dependent signal is novel and important, and that is the take home message of this paper.

1) The paper is complex and some of the "side issues" (such as the interplay between ATM and ATR signalling) are difficult to interface with the ATM analysis. Additionally, the role of Kap1 remains somewhat tentative yet appears more than correlatory given the phenotype of the S824A mutant. So it is difficult to grasp whether this is one of the "take-home" messages - solid or just tentative. Hence it is important to highlight the take home messages clearly.

2) It is unclear whether this mechanism regulates mitotic entry after low (physiologically relevant) doses or predominantly describes the "adaptation" process, which represents a mechanism to allow cell cycle entry even when substantial levels of DSBs remain. Because NCS is used it is difficult to assess the magnitude of damage but for most experiments this seems to be the equivalent of using high IR doses (since the duration of arrest is often 20-30 h). To explain more: some studies (eg Deckbar et al., referenced) have looked at mitotic entry after physiologically relevant doses of X-rays (ie with entry occurring after 3-5 h) - or at even lower doses (where checkpoints are not activated). Other studies (eg Syljuåsen RG et al: PMID: 17079442) have examined checkpoint adaptation - which occurs after much higher doses. The discussion here relates to both these studies but it is unclear if the mechanism proposed holds only for adaptation or for lower doses. At least one experiment should be done to show that the same mechanism regulates mitotic entry after a low dose (when arrest is only a few hours). This may be difficult if the ATM sensor signal is insensitive at low doses but the Plk1 signal could be examined (and the roles of ATM, ATR and Wee1). The goal is simply to say that this phenomenon holds for lower doses where survival occurs and is important for the discussion about low doses.

3) Given the high doses (and long duration of arrest), these studies differ from some of the other studies in that whilst the cells entering mitotis first may represent cells in G2 when irradiated, the

duration of arrest will be predominantly controlled by cells that have traversed S phase with damage. Even NCS induces many SSBs - but DSBs may also be encountered the fork (the S phase checkpoint is less strong and irradiated S phase cells also arrest at the G2/M checkpoint). The regulation of entry of these cells may be more influenced by ATR activation at stalled forks rather than following resection of DSBs. Thus, it is difficult to assess if the role of ATR is a consequence of the prolonged arrest cells receiving damage in G2 phase or S phase damaged cells. This is perhaps not central to the model for ATM regulation but is important at least to discuss (since currently it is entirely suggested that resection at DSBs occurs). To determine the distinction, could the analysis be carried out in cells where Edu is added immediately prior to NCS treatment. The release could then be examined in Edu positive (S phase when treated) versus Edu negative (presumeably G2 phase when treated) cells.

4) A confusing aspect is that the entire focus is on ATM's regulation of Plk1 - yet ATM also regulates Chk2 and ATR, Chk1 and it is known that Chk2 or Chk1 inhibitors abrogate G2/M checkpoint arrest. If I understand correctly, the model proposes that Plk1 is the rate limiting factor determining the duration of arrest and that Chk1/2 function upstream? At the least the roles of Chk2/Chk1 should be discussed. Could a Plk1 inhibitor be used to consolidate its specific role - if Plk1 only regulates the duration of arrest, Plki treated cells should initiate arrest normally but fail to emerge from it. However, the role of Wee1 has to be considered in both these components regulating checkpoint arrest.

5) It is stated that inhibiting DSB repair prolongs checkpoint arrest and this is modelled using PARP inhibition and RNF8 siRNA. This is important and I feel should be backed up by direct evidence, not least because it is unclear how this modelling was done. Whilst RNF8 should cause a small repair defect, it is not clear that PARPi gives a repair defect in G2. Indeed, PARPi place a reliance on HR (and is dispensable for NHEJ) - and I am not aware of any studies showing a DSB repair defect in G2 cells treated with PARPi. Such treatment may, in fact, affect lesions forming following replication, but this represents a somewhat distinct model. The impact of these two treatments should be directly examined and if PARPi does allow prolonged arrest, this should be further evaluated. siRNA Rad51 or another HR factor may be a better way to impede DSB repair in G2.

Referee #2

(Report for Author)
In this study Jaiswal et al., constructed two reporters in order to monitor the activity of DNA damage response (DDR) in living cells. They show that the generated reporters, although initially constructed to report ATM- and ATR-mediated activity, they specifically respond to ATM activation. In order to be able to monitor ATM activity at different subcellular locations, they constructed one chromatin bound and one diffusible reporter. These reporters showed different kinetics of ATM activity upon NCS treatment, but surprisingly the authors didn't correlate their response with the kinetics of known targets of ATM upon NCS (see below). By using a similar reporter that probes Plk1 activity, they correlated Plk1 activation with dephosphorylation of the chromatin bound reporter upon NCS treatment. Using the chromatin bound ATM reporter the authors showed that, upon NCS or localized DNA damage, ATM activity is detected throughput the chromatin. Surprisingly however they didn't detect an enrichment of ATM activation at DNA damage foci or at laser-induced damage sites, where, clearly, known targets of ATM (including ATM autophosphorylation, Figure 6D, or pSMC3, SupplFigure 8A) are enriched. They nicely showed that Wip1 phosphatase can counteract ATM activity on chromatin and they assembled a mathematical model that allowed to predict that ATM activity on chromatin enforces a checkpoint arrest above a threshold of damage and ensures a minimal duration of the checkpoint.

In general the study aims at understanding of how cell cycle could restart before completion of DNA damage, an interesting topic that has implications for understanding the mechanism of checkpoint adaptation in human cells. Although the findings presented in this manuscript are interesting, it is difficult to assess the quality of the observations as statistics are missing across the study. In addition the characterization of the main tools of the study, the two reporters, is rather weak (see below) and the effect of ATM activity on the duration of the cell cycle is rather correlative. Assessing the criticism raised as major and minor points below will substantially improve the manuscript, before consideration for publication in EMBOJ.

Major comments:

I would expect an early in the study direct comparison of the kinetics of targets of ATM upon damage (e.g NCS or laser induced damage) such as Kap1-S824, SMC-S1083 or ATM autophosphorylation with the kinetics of both the immobilized onto chromatin or the soluble ATKAR reporter. This is a crucial experiment which will help the authors decide which of the two reporters can be used to accurately follow ATM activity in live cells. This is an important point since: 1) the reporters show different responses upon NCS treatment and 2) the authors could not detect an enrichment of H2B-ATKAR activity on DNA damage foci (while both gH2AX and pS1981 ATM staining persisted), suggesting that the chromatin bound reporter actually does not report the ATM-dependent phosphorylation events that take place onto chromatin (e.g the early DDR activation events). Similarly, signal from the same reporter started to revert within an hour after the NCS addition, which, as the authors mentioned, suggests that "the ATM-mediated phosphorylation of an artificial substrate becomes less efficient when the substrate is targeted to chromatin". However, in most of the experiments in the study the same chromatin-bound reporter was used. I would suggest that the authors perform a direct comparison of the activity of their reporters and known ATM targets on chromatin and nucleoplasm.
I was also rather disappointed to see that across the manuscript there was not a single experiment with the appropriate statistics. Especially in live experiments where the number of the reported analyzed cells was 10-20, it is very difficult to estimate the significance of the findings.

Minor:

Although the main conclusions of the study are drawn based on the ATM/ATR Activity reporters the authors haven't explained how these reporter's actually work. Including a scheme of the reporters would help readers not familiar with the FRET-based methodologies to understand how these reporters in principle report ATM-dependent phosphorylation.
How the reporters respond when cells are pretreated with ATMi inhibitor before the induced DNA damage (e.g laser-induced, NCS).

Referee #3

(Report for Author)
Comment on the modeling part of the manuscript "ATM/Wip1 activities at chromatin control Plk1 re-activation to determine G2 checkpoint duration [EMBOJ-2016-96082]"

In this manuscript, the authors studies the cell cycle response to DNA damage. DNA damage activated the kinase ATM, which phosphorylate their targets to block cell cycle progression. On top of this, the counter acting phosphatase Wip1 dephosphorylate the target proteins of ATM. Hence, the target proteins could be de-phosphorylated and re-activated before the complete inactivation of the kinases ATM. As a result, the cell cycle progression is able to continue in the presence of residual DNA damage and ATM activities. Overall, this is a logical story that makes intuitive sense.

In the manuscript, the authors also incorporated a simple mathematical model. The current model has some flaws:

One: the current model is oversimplified and does not take into consideration of the biological details of the DNA damage checkpoint. Given that detailed mathematical models have been constructed on the DNA damage checkpoint, it is better to use and modify these detailed models rather than constructing an over simplified version.

Two: the model parameters should be constrained by biological data, and the model simulation should be compared to the experimental observations so that the model can be integrated to the experimental work in this manuscript and the data from the literature.

Three: the model prediction, that there is a threshold of DNA damage, should be properly analyzed with nonlinear tools such as one parameter bifurcation analysis. Furthermore, whether such a threshold functions in the control of cell cycle checkpoint should be tested within the experimental

framework proposed in this manuscript.

In conclusion, the reviewer suggests that the current mathematical model should be greatly improved following the above suggestions. The end result might then be an impactful manuscript combining experimental and theoretical work that is suitable for the publication on EMBOJ.

Authors' response 21 December 2016

Thank you very much for your email. I have today discussed the reviewers' comments with some of the co-authors and we were happy to note that the reviewers seem generally positive regarding our main findings and that the comments were constructive. We concluded that we from our side would like to proceed to prepare a resubmission.

As you suggested, I have prepared a preliminary draft for a point-by point response. The attached draft may lack some polish and politeness, and is not intended as a direct response to the reviewers, but more as an indication on how we see the main lines of reasoning. At the last page, I have assembled a list of the experiments currently in the reasoning. Some of these experiments may be more important than others, and the time-line for a possible re-submission will depend on whether to perform all these experiments. Preliminary, I think the experiments can be performed in less than three months.

Would it be possible to discuss the proposed approach on the phone? In that case, when would it suit you to talk?

POINT-BY-POINT RESPONSE

Referee #1

(Report for Author)
This paper uses a Fret based sensor to monitor free nuclear or chromatin bound ATM activity to assess the regulation of the G2/M checkpoint after DSB induction, with the focus being on Plk1 activity. It is shown that Plk1 activity after NCS decreases and then returns and that loss of the chromatin bound ATM sensor signal (but not free nuclear signal) correlates with the increase in Plk1 activity, which precedes mitotic entry. It is concluded that a genome wide chromatin bound ATM signal regulates the initial decrease in Plk1 activity and its subsequent return. The ATM signal is also influenced by Wip1, leading to the model that a chromatin bound ATM/Wip1 regulated signal determines the initial duration of checkpoint arrest. However, ATR also influences the rate of return of Plk1 activity ie its influence arises at later times after DSB induction (achieved using NCS). At face value this is consistent with the notion that DSBs undergo resection in G2 phase leading to a switch from ATM to ATR activity. Interestingly, however, mitotic entry arises even in the presence of ATR inhibitors, suggesting that it cannot fully prevent entry. Previous studies have shown that mitotic entry occurs prior to the completion of DSB repair and this is consolidated here showing that when Plk1 activity returns and mitotic entry occurs, gH2AX foci, active ATM at foci and non-chromatin bound ATM activity persist. Thus, the model proposed is that a chromatin bound ATM/Wip-counteracted signal causes an initial decrease in Plk1 activity, which is then sustained by ATR activity. This is distinct to a model whereby ATM (and later ATR) activity at DSBs regulates the duration of checkpoint arrest. It is suggested that this allows ATM's function in DSB repair to be sustained even though mitotic re-entry can occur. Kap1 is chromatin bound and phosphorylated by ATM and the duration of its phosphorylation correlates with the return of Plk1 activity. This represents a complex but interesting paper and, importantly, provides evidence for a novel mechanism for the regulation of mitotic entry after DSB induction. Although there are aspects that appear unclear (discussed below), the notion that the mitotic entry signal is regulated by a pan nuclear chromatin bound ATM dependent signal is novel and important, and that is the take home message of this paper.

1) The paper is complex and some of the "side issues" (such as the interplay between ATM and ATR signalling) are difficult to interface with the ATM analysis. Additionally, the role of Kap1 remains somewhat tentative yet appears more than correlatory given the phenotype of the S824A

mutant. So it is difficult to grasp whether this is one of the "take-home" messages - solid or just tentative. Hence it is important to highlight the take home messages clearly.

We will re-formulate parts of the manuscript to highlight the take home message.

2) It is unclear whether this mechanism regulates mitotic entry after low (physiologically relevant) doses or predominantly describes the "adaptation" process, which represents a mechanism to allow cell cycle entry even when substantial levels of DSBs remain. Because NCS is used it is difficult to assess the magnitude of damage but for most experiments this seems to be the equivalent of using high IR doses (since the duration of arrest is often 20-30 h). To explain more: some studies (eg Deckbar et al., referenced) have looked at mitotic entry after physiologically relevant doses of X-rays (ie with entry occurring after 3-5 h) - or at even lower doses (where checkpoints are not activated). Other studies (eg Syljuåsen RG et al: PMID: 17079442) have examined checkpoint adaptation - which occurs after much higher doses. The discussion here relates to both these studies but it is unclear if the mechanism proposed holds only for adaptation or for lower doses. At least one experiment should be done to show that the same mechanism regulates mitotic entry after a low dose (when arrest is only a few hours). This may be difficult if the ATM sensor signal is insensitive at low doses but the Plk1 signal could be examined (and the roles of ATM, ATR and Wee1). The goal is simply to say that this phenomenon holds for lower doses where survival occurs and is important for the discussion about low doses.

We will film the Plk1 FRET signal in the presence or absence of ATM, ATR, and Wee1 inhibitors, while titrating down the dose of NCS used. However, although experimentally feasible, there are concerns among some authors that introducing Wee1 will unnecessarily complicate the manuscript.

3) Given the high doses (and long duration of arrest), these studies differ from some of the other studies in that whilst the cells entering mitotis first may represent cells in G2 when irradiated, the duration of arrest will be predominantly controlled by cells that have traversed S phase with damage. Even NCS induces many SSBs - but DSBs may also be encountered the fork (the S phase checkpoint is less strong and irradiated S phase cells also arrest at the G2/M checkpoint). The regulation of entry of these cells may be more influenced by ATR activation at stalled forks rather than following resection of DSBs. Thus, it is difficult to assess if the role of ATR is a consequence of the prolonged arrest cells receiving damage in G2 phase or S phase damaged cells. This is perhaps not central to the model for ATM regulation but is important at least to discuss (since currently it is entirely suggested that resection at DSBs occurs). To determine the distinction, could the analysis be carried out in cells where Edu is added immediately prior to NCS treatment. The release could then be examined in Edu positive (S phase when treated) versus Edu negative (presumeably G2 phase when treated) cells.

We agree that there may be multiple routes to ATR activation after NCS and are happy to discuss them in a revised manuscript.
We also agree that in the experiments performed on unsynchronized cells, at least some of the recovering cells may have been in S-phase upon NCS addition. We will test this by pulsing EdU before NCS addition and analyse recovering cells trapped in mitotis for EdU content by FACS. If unsuccessful, we will use live-cell markers for cell cycle phase and track cells in films from NCS addition to mitosis after checkpoint recovery. However, we would like to stress that whether cells are initially hit by NCS in S or G2 phase does not change any of our main conclusions in the manuscript.

4) A confusing aspect is that the entire focus is on ATM's regulation of Plk1 - yet ATM also regulates Chk2 and ATR, Chk1 and it is known that Chk2 or Chk1 inhibitors abrogate G2/M checkpoint arrest. If I understand correctly, the model proposes that Plk1 is the rate limiting factor determining the duration of arrest and that Chk1/2 function upstream? At the least the roles of Chk2/Chk1 should be discussed. Could a Plk1 inhibitor be used to consolidate its specific role - if Plk1 only regulates the duration of arrest, Plki treated cells should initiate arrest normally but fail to emerge from it. However, the role of Wee1 has to be considered in both these components regulating checkpoint arrest.

In preliminary discussions, not all authors agreed on how to interpret this point. We are happy to increase the discussion on Chk1/Chk2. Similarly, we are happy to add Plk1 inhibitor and assess the

consequence on checkpoint duration – this is a straightforward experiment and already shown in many publications. We could also assess whether addition of a Plk1 inhibitor affects the rate of decrease of ATM activity. However, there were large discussions among co-authors whether such an experiment would be meaningful and relevant for the manuscript.

We would caution against calling Chk1/Chk2 upstream of Plk1. As we indicate in the schematics depicting our model (Figure 7A), Plk1 is both upstream and downstream of Chk1. We have chosen to simplify the large network of proteins to two large interlinked feedback-loops: one positive, centering around Cdk and Plk1, and one negative, centering around ATR-Chk1 on one side and Cdk-Plk1 on the other. We are happy to introduce the role of Wee1 as an inhibitor of Cdk and a target of Chk1, Plk1 and Cdk1 in the introduction, but believe that bringing in all players in the model on figure 7 will decrease the readability and accessibility of our findings. We will extend and reformulate sections on the model to increase clarity.

5) It is stated that inhibiting DSB repair prolongs checkpoint arrest and this is modelled using PARP inhibition and RNF8 siRNA. This is important and I feel should be backed up by direct evidence, not least because it is unclear how this modelling was done. Whilst RNF8 should cause a small repair defect, it is not clear that PARPi gives a repair defect in G2. Indeed, PARPi place a reliance on HR (and is dispensable for NHEJ) - and I am not aware of any studies showing a DSB repair defect in G2 cells treated with PARPi. Such treatment may, in fact, affect lesions forming following replication, but this represents a somewhat distinct model. The impact of these two treatments should be directly examined and if PARPi does allow prolonged arrest, this should be further evaluated. siRNA Rad51 or another HR factor may be a better way to impede DSB repair in G2.

We agree that there may be better perturbations than PARP inhibition. We will transfect siRNA for additional HR and NHEJ factors and quantify H2B-ATKAR FRET ratio over time. Further, the model prediction on a DNA damage threshold will be extended, both in response to this comment and to the comments of reviewer 3.

Referee #2

(Report for Author)
In this study Jaiswal et al., constructed two reporters in order to monitor the activity of DNA damage response (DDR) in living cells. They show that the generated reporters, although initially constructed to report ATM- and ATR-mediated activity, they specifically respond to ATM activation. In order to be able to monitor ATM activity at different subcellular locations, they constructed one chromatin bound and one diffusible reporter. These reporters showed different kinetics of ATM activity upon NCS treatment, but surprisingly the authors didn't correlate their response with the kinetics of known targets of ATM upon NCS (see below). By using a similar reporter that probes Plk1 activity, they correlated Plk1 activation with dephosphorylation of the chromatin bound reporter upon NCS treatment. Using the chromatin bound ATM reporter the authors showed that, upon NCS or localized DNA damage, ATM activity is detected throughput the chromatin. Surprisingly however they didn't detect an enrichment of ATM activation at DNA damage foci or at laser-induced damage sites, where, clearly, known targets of ATM (including ATM autophosphorylation, Figure 6D, or pSMC3, SupplFigure 8A) are enriched. They nicely showed that Wip1 phosphatase can counteract ATM activity on chromatin and they assembled a mathematical model that allowed to predict that ATM activity on chromatin enforces a checkpoint arrest above a threshold of damage and ensures a minimal duration of the checkpoint.
In general the study aims at understanding of how cell cycle could restart before completion of DNA damage, an interesting topic that has implications for understanding the mechanism of checkpoint adaptation in human cells. Although the findings presented in this manuscript are interesting, it is difficult to assess the quality of the observations as statistics are missing across the study. In addition the characterization of the main tools of the study, the two reporters, is rather weak (see below) and the effect of ATM activity on the duration of the cell cycle is rather correlative. Assessing the criticism raised as major and minor points below will substantially improve the manuscript, before consideration for publication in EMBOJ.

Major comments:

I would expect an early in the study direct comparison of the kinetics of targets of ATM upon damage (e.g NCS or laser induced damage) such as Kap1-S824, SMC-S1083 or ATM autophosphorylation with the kinetics of both the immobilized onto chromatin or the soluble ATKAR reporter. This is a crucial experiment which will help the authors decide which of the two reporters can be used to accurately follow ATM activity in live cells. This is an important point since: 1) the reporters show different responses upon NCS treatment and 2) the authors could not detect an enrichment of H2B-ATKAR activity on DNA damage foci (while both gH2AX and pS1981 ATM staining persisted), suggesting that the chromatin bound reporter actually does not report the ATM-dependent phosphorylation events that take place onto chromatin (e.g the early DDR activation events). Similarly, signal from the same reporter started to revert within an hour after the NCS addition, which, as the authors mentioned, suggests that "the ATM-mediated phosphorylation of an artificial substrate becomes less efficient when the substrate is targeted to chromatin". However, in most of the experiments in the study the same chromatin-bound reporter was used. I would suggest that the authors perform a direct comparison of the activity of their reporters and known ATM targets on chromatin and nucleoplasm.

We will address this point by fractionation and WB at different times after NCS addition. We will also fix cells at different time points after NCS addition to perform quantitative IF for ATM targets, in order to get a more close comparison of the phosphorylation dynamics of a selection of ATM targets and ATKAR and H2B-ATKAR.

We would like to stress that we in figure 8A show that dephosphorylation of different endogenous ATM targets follow different dynamics. We provide an explanation for this phenomenon by showing that an artificial ATM target is dephosphorylated differently depending on its intranuclear localization. We therefore do not agree to the statement "decide which of the two reporters can be used to accurately follow ATM activity in live cells". This is not least since we show examples of ATM targets (ATM p1981, gH2AX) that similar to ATKAR are retained until late in G2, and the ATM target KAP1, that similar to H2B-ATKAR is dephosphorylated before Plk1 activity resumes. Further, endogenous targets show different localization: whereas gH2AX largely remain at DDR foci and is sustained long after damage, pKAP1 is localized throughout chromatin. Thus, it is in our opinion not meaningful to say that one of the probes is more accurate than the other. Endogenous ATM targets follow different dynamics and we here provide a mechanism that at least partly can explain this phenomenon.

We do not detect H2B-ATKAR phosphorylation at DDR foci, which could be due to several reasons. First, the amount of H2B-ATKAR may be low on DDR focus compared to H2B-ATKAR on surrounding chromatin in same pixel. In this sense, detection of a ratio-change is less efficient than detection of enrichment of a tagged protein whereas the technical imaging challenges are higher. In addition, during resection or remodeling, the amount of nucleosomes in DDR foci containing H2B-ATKAR may be reduced. Further, despite significant efforts from several labs, including ours, no one has to our knowledge succeeded in detecting FRET-change on a probe targeted to the centrosome. These negative data are not published, but are generally believed to depend on FRET-probes being restricted to change conformation in the dense cross-linked protein environment surrounding the centrosome. It is very possible that DDR foci contain similar protein environments, thus making FRET changes difficult to study there. Nonetheless, we show that outside DDR foci the ATM responsive H2B-ATKAR follows similar desphosphorylation dynamics as the endogenous ATM target pKAP1. As H2B-ATKAR dephosphorylation, but not ATKAR dephosphorylation, correlated to when Plk1 activity resumed, we in this manuscript focused mainly on studying H2B-ATKAR.

I was also rather disappointed to see that across the manuscript there was not a single experiment with the appropriate statistics. Especially in live experiments where the number of the reported analyzed cells was 10-20, it is very difficult to estimate the significance of the findings.

We agree and will introduce statistical tests.

Minor:

Although the main conclusions of the study are drawn based on the ATM/ATR Activity reporters the authors haven't explained how these reporter's actually work. Including a scheme of the reporters would help readers not familiar with the FRET-based methodologies to understand how these reporters in principle report ATM-dependent phosphorylation.

We agree and will include a scheme

How the reporters respond when cells are pretreated with ATMi inhibitor before the induced DNA damage (e.g laser-induced, NCS).

These data are already present in the manuscript (Figures 1A and S1E for NCS, and figure 5C for laser-microirradiation)

Referee #3

(Report for Author)
Comment on the modeling part of the manuscript
"ATM/Wip1 activities at chromatin control Plk1 re-activation to determine G2 checkpoint duration [EMBOJ-2016-96082]"

In this manuscript, the authors studies the cell cycle response to DNA damage. DNA damage activated the kinase ATM, which phosphorylate their targets to block cell cycle progression. On top of this, the counter acting phosphatase Wip1 dephosphorylate the target proteins of ATM. Hence, the target proteins could be de-phosphorylated and re-activated before the complete inactivation of the kinases ATM. As a result, the cell cycle progression is able to continue in the presence of residual DNA damage and ATM activities. Overall, this is a logical story that makes intuitive sense.

In the manuscript, the authors also incorporated a simple mathematical model. The current model has some flaws:

One: the current model is oversimplified and does not take into consideration of the biological details of the DNA damage checkpoint. Given that detailed mathematical models have been constructed on the DNA damage checkpoint, it is better to use and modify these detailed models rather than constructing an over simplified version.

We respectfully disagree. We would argue that both the more detailed holistic models and the simplified reductionist models have their pros and cons. There are numerous examples in the literature in which simplistic models can be used to verify experimental discoveries and predict phenomena (e.g. for localization feedback in late G2: Santos et al, Cell 2012 www.ncbi.nlm.nih.gov/pubmed/22726437). The model we devised is not intended to capture every detail, but due to the low number of parameters, to robustly show insight into major mechanisms concerning how recovery from a G2 checkpoint is regulated.

As reviewer one commented: "This represents a complex but interesting paper and, importantly, provides evidence for a novel mechanism for the regulation of mitotic entry after DSB induction". We believe that the model we devised both functions as a framework to explain our findings and as a mean to both verify and predict the general consequences of our experimental findings.

We do agree that a detailed model would allow additional analyses. However, to be accurate, such a model would need to incorporate DDR foci, chromatin, nucleoplasm and cytoplasm and a large number of constants that currently are unknown would need to be determined. It is our long-term goal to assemble a data-driven functional model with spatial constraints, and we hope that the insights, tools, and data we developed in this study will form a base for such efforts.

Two: the model parameters should be constrained by biological data, and the model simulation should be compared to the experimental observations so that the model can be integrated to the experimental work in this manuscript and the data from the literature.

We are happy to constrain parameters and compare simulations to experimental data. However, we believe that one of the strengths of the current model is that constants are restricted to 1, 2, and 10, thus providing robustness for the analysis. We therefore prefer to also retain the current model.

Three: the model prediction, that there is a threshold of DNA damage, should be properly analyzed with nonlinear tools such as one parameter bifurcation analysis. Furthermore, whether such a threshold functions in the control of cell cycle checkpoint should be tested within the experimental framework proposed in this manuscript.

We thank the reviewer for the suggestion. We will run a bifurcation analysis to determine the threshold and compare that to experimental data. The experimental data on H2B-ATKAR phosphorylation after inhibiting DNA repair will be expanded to better characterize the effects of sustained DNA damage, both in response to this comment and to a comment of reviewer 1.

In conclusion, the reviewer suggests that the current mathematical model should be greatly improved following the above suggestions. The end result might then be an impactful manuscript combining experimental and theoretical work that is suitable for the publication on EMBOJ.

Experiments:

- Film the Plk1 FRET signal in the presence or absence of ATM, ATR, and Wee1 inhibitors, while titrating down the dose of NCS used.
- Pulsing EdU before NCS addition and analyze recovering cells trapped in mitosis for EdU content by FACS. If unsuccessful, we will use live-cell markers for cell cycle phase and track cells in films from NCS addition to mitosis after checkpoint recovery.
- Add Plk1 inhibitor and assess the consequence on checkpoint duration – this is a straightforward experiment and already shown in many publications. We could also assess whether addition of a Plk1 inhibitor affects the rate of decrease of ATM activity.
- Transfect siRNA for additional HR and NHEJ factors and quantify H2B-ATKAR FRET ratio over time.
- Fractionation and WB at different times after NCS addition.
- Fix cells at different time points after NCS addition to perform quantitative IF for ATM targets

Modeling: constrain parameters and compare simulations to the current model, and also to compare simulations to experimental data as supplementary data.

Modeling: run a bifurcation analysis

Other:

Introduce statistical tests
Introduce schematic figure of probe

1st Editorial Decision 22 December 2016

Thank you again for sending in and talking me through your tentative response to the referee comments on your recent submission, EMBOJ-2016-96082. I am pleased to see that you appear to be in a good position to satisfactorily address the points raised by the referees, and would therefore like to formally invite you to revise the manuscript along the lines proposed in your letter and discussed in our conversation.

In particular, it will be important to strengthen the mathematical modeling in response to referee 1 and referee 3's points 2 and 3; while providing a more holistic model based on numerous uncertain parameters is not necessary at this stage.

Please consider subtle improvements of narrative, discussion and schematic presentation in response to referees 1 and 2, as proposed. Regarding statistical analyses, please carefully go through the figures and make sure that the nature of the depicted error bars is always clearly stated in the figure

legend; dedicated statistical tests may only be needed for panels where the presentation is not by itself already intuitively meaningful.

With regard to further experimental analyses, it would be important to attempt RNAi interference with additional HR/NHEJ factors (ref 1 point 5), to test Plk1 FRET in NCS titration (without need to incorporate additional tests of the downstream component Wee1) (ref 1 point 2), and to provide the comparative time course analysis of different ATM targets after NCS addition (in response to ref 2). Furthermore, it would be helpful if referee 1's third point could be tested at least by the proposed EdU pulsing experiment and FACS analysis, and if the effect of Plk1 inhibition on the rate of ATM activity decrease (in response to ref 1 point 4) could be assessed.

Thank you again for the opportunity to consider this work for The EMBO Journal, and I look forward to your revision.

1st Revision - authors' response 05 April 2017

Please find enclosed our revised version of the manuscript EMBOJ-2016-96082 entitled "ATM/Wip1 activities at chromatin control Plk1 re-activation to determine G2 checkpoint duration."
We have performed experiments to address reviewer concerns as outlined in our earlier correspondence. In particular, we have adapted our model to experimental data and tested predictions based on decreasing amounts of DNA damage. We have further more solidly defined the notion of a threshold of ATM activity for Plk1 re-activation, and now show that both H2B-ATKAR phosphorylation and a checkpoint are sustained after preventing completion of DNA repair by CtIP RNAi or DNA-PK inhibition. We have also performed experiments to assess the role of ATR in G2 cells and now show that cells receiving DNA damage in either S or G2 phase eventually can recover from a checkpoint and enter mitosis. In response to reviewer 2, we further use a time-course combined with cell fractionation to study phosphorylation of endogenous ATM targets.

We believe that the revision process has improved the manuscript and hope that you agree that it is now suitable for publication in EMBO journal.

POINT-BY-POINT RESPONSE

We thank the reviewers for the constructive assessment, which in our opinion has improved the manuscript. Major changes to the manuscript are indicated in red.

New Figure panels:

1A Schematics of FRET probe

7C Steady-state analysis of model

7D H2B-ATKAR response and mitotic entry after CtIP RNAi, RNF8 RNAi, or DNA-PK inhibition

EV3A H2B-ATKAR response and mitotic entry after Plk1 inhibition

EV3B Optimization of model according to experimental data

EV3C Optimized model

EV3D Simulation of checkpoint recovery after low levels of DNA damage

EV3E Plk1 FRET in recovering cells after low levels of DNA damage

EV3F Effect of ATM or ATR inhibition on cells receiving low amounts of DNA damage in G2 phase

EV3G Simulation of checkpoint recovery at different starting activities of Plk1

EV3H Both S and G2 cells exposed to NCS recover and enter mitosis

EV4A Fractionation and time-course of endogenous ATM targets

Referee #1

(Report for Author)
This paper uses a Fret based sensor to monitor free nuclear or chromatin bound ATM activity to assess the regulation of the G2/M checkpoint after DSB induction, with the focus being on Plk1 activity. It is shown that Plk1 activity after NCS decreases and then returns and that loss of the chromatin bound ATM sensor signal (but not free nuclear signal) correlates with the increase in Plk1 activity, which precedes mitotic entry. It is concluded that a genome wide chromatin bound ATM signal regulates the initial decrease in Plk1 activity and its subsequent return. The ATM signal is also influenced by Wip1, leading to the model that a chromatin bound ATM/Wip1 regulated signal determines the initial duration of checkpoint arrest. However, ATR also influences the rate of return of Plk1 activity i.e. its influence arises at later times after DSB induction (achieved using NCS). At face value this is consistent with the notion that DSBs undergo resection in G2 phase leading to a switch from ATM to ATR activity. Interestingly, however, mitotic entry arises even in the presence of ATR inhibitors, suggesting that it cannot fully prevent entry. Previous studies have shown that mitotic entry occurs prior to the completion of DSB repair and this is consolidated here showing that when Plk1 activity returns and mitotic entry occurs, gH2AX foci, active ATM at foci and non-chromatin bound ATM activity persist. Thus, the model proposed is that a chromatin bound ATM/Wip-counteracted signal causes an initial decrease in Plk1 activity, which is then sustained by ATR activity. This is distinct to a model whereby ATM (and later ATR) activity at DSBs regulates the duration of checkpoint arrest. It is suggested that this allows ATM's function in DSB repair to be sustained even though mitotic re-entry can occur. Kap1 is chromatin bound and phosphorylated by ATM and the duration of its phosphorylation correlates with the return of Plk1 activity. This represents a complex but interesting paper and, importantly, provides evidence for a novel mechanism for the regulation of mitotic entry after DSB induction. Although there are aspects that appear unclear (discussed below), the notion that the mitotic entry signal is regulated by a pan nuclear chromatin bound ATM dependent signal is novel and important, and that is the take home message of this paper.

We thank the reviewer for the constructive comments.

1) The paper is complex and some of the "side issues" (such as the interplay between ATM and ATR signalling) are difficult to interface with the ATM analysis. Additionally, the role of Kap1 remains somewhat tentative yet appears more than correlatory given the phenotype of the S824A mutant. So it is difficult to grasp whether this is one of the "take-home" messages - solid or just tentative. Hence it is important to highlight the take home messages clearly.

We have reformulated parts of the manuscript to highlight the take home message, which is that ATM activity on chromatin controls Plk1 activation. We agree that the manuscript is complex, as we have tried to address how ATM affects the duration of a G2 arrest. We believe our experiments show that both ATR and Plk1 play key roles in this process, which is important for our conclusion. All three proteins function in the context of many other proteins, as Chk2, Chk1, and Wee1, and in our model we have tried to reduce complexity by treating the groups of proteins as functional entities. We have expanded the description of our reasoning to select these functional entities on page 10. We find that Kap1 has at least some function for determining the duration of a checkpoint arrest. However, Kap1 is likely only one out of several ATM targets, and we are throughout the manuscript careful not to overstate its importance. To highlight the take-home message, we have included a suggestion for synopsis:

- *ATKAR: a FRET-based probe for ATM and ATR activity in live cells*
- *DNA damage-induced spread of ATM activity across chromatin prevents Plk1 activation*
- *ATM activity is counteracted by the chromatin-bound phosphatase Wip1*
- *Plk1 activation can be initiated despite active ATM at DDR foci*

2) It is unclear whether this mechanism regulates mitotic entry after low (physiologically relevant) doses or predominantly describes the "adaptation" process, which represents a mechanism to allow cell cycle entry even when substantial levels of DSBs remain. Because NCS is used it is difficult to assess the magnitude of damage but for most experiments this seems to be the equivalent of using high IR doses (since the duration of arrest is often 20-30 h). To explain more: some studies (eg Deckbar et al., referenced) have looked at mitotic entry after physiologically relevant doses of X-rays (ie with entry occurring after 3-5 h) - or at even lower doses (where checkpoints are not activated). Other studies (eg Syljuåsen RG et al: PMID: 17079442) have examined checkpoint adaptation - which occurs after much higher doses. The discussion here relates to both these studies but it is unclear if the mechanism proposed holds only for adaptation or for lower doses. At least one experiment should be done to show that the same mechanism regulates mitotic entry after a low dose (when arrest is only a few hours). This may be difficult if the ATM sensor signal is insensitive at low doses but the Plk1 signal could be examined (and the roles of ATM, ATR and Wee1). The goal is simply to say that this phenomenon holds for lower doses where survival occurs and is important for the discussion about low doses.

We agree that this is a very relevant point and have now included a paragraph on page 11 describing the effect of lower level of DNA damage. We combine experiments (Figure EV3E and F) with modeling (Figure EV3D, E, and G) and come to the conclusion that the same framework applies, although ATM activity may not be sufficient to completely reset Plk1 activity when the amount of damage is low.

3) Given the high doses (and long duration of arrest), these studies differ from some of the other studies in that whilst the cells entering mitotis first may represent cells in G2 when irradiated, the duration of arrest will be predominantly controlled by cells that have traversed S phase with damage. Even NCS induces many SSBs - but DSBs may also be encountered the fork (the S phase checkpoint is less strong and irradiated S phase cells also arrest at the G2/M checkpoint). The regulation of entry of these cells may be more influenced by ATR activation at stalled forks rather than following resection of DSBs. Thus, it is difficult to assess if the role of ATR is a consequence of the prolonged arrest cells receiving damage in G2 phase or S phase damaged cells. This is perhaps not central to the model for ATM regulation but is important at least to discuss (since currently it is entirely suggested that resection at DSBs occurs). To determine the distinction, could the analysis be carried out in cells where Edu is added immediately prior to NCS treatment. The release could then be examined in Edu positive (S phase when treated) versus Edu negative (presumeably G2 phase when treated) cells.

We completely agree to this point. We performed the experiment as suggested, and in Figure EV3H we now show that both G2 and S-phase cells recover in our setup. Importantly, we further show that inhibition of ATR stimulates recovery of cells that received DNA damage in G2 phase (Figure EV3F). While we agree that the routes to ATR activation may be complex in S-phase, this shows that ATR activity after NCS treatment cannot be explained by stalled replication forks alone.

4) A confusing aspect is that the entire focus is on ATM's regulation of Plk1 - yet ATM also regulates Chk2 and ATR, Chk1 and it is known that Chk2 or Chk1 inhibitors abrogate G2/M checkpoint arrest. If I understand correctly, the model proposes that Plk1 is the rate limiting factor determining the duration of arrest and that Chk1/2 function upstream? At the least the roles of Chk2/Chk1 should be discussed. Could a Plk1 inhibitor be used to consolidate its specific role - if Plk1 only regulates the duration of arrest, Plki treated cells should initiate arrest normally but fail to emerge from it. However, the role of Wee1 has to be considered in both these components regulating checkpoint arrest.

In new Figure EV3A, we now show that H2B-ATKAR signal is not affected by addition of a Plk1 inhibitor, whereas mitotic entry after NCS addition is delayed.

We would caution against calling Chk1/Chk2 upstream of Plk1. As we indicate in the schematics depicting our model (Figure 7A), Plk1 is both upstream and downstream of Chk1. We have chosen to simplify the large network of proteins to two large interlinked feedback-loops: one positive, centering around Cdk and Plk1, and one negative, centering around ATR-Chk1 on one side and Cdk-Plk1 on the other. On page 10 we have now extended the description of the model and mention

Chk1, Chk2, and Wee1, but believe that bringing in more players in the model on figure 7 will decrease the readability and accessibility of our findings.

5) It is stated that inhibiting DSB repair prolongs checkpoint arrest and this is modelled using PARP inhibition and RNF8 siRNA. This is important and I feel should be backed up by direct evidence, not least because it is unclear how this modelling was done. Whilst RNF8 should cause a small repair defect, it is not clear that PARPi gives a repair defect in G2. Indeed, PARPi place a reliance on HR (and is dispensable for NHEJ) - and I am not aware of any studies showing a DSB repair defect in G2 cells treated with PARPi. Such treatment may, in fact, affect lesions forming following replication, but this represents a somewhat distinct model. The impact of these two treatments should be directly examined and if PARPi does allow prolonged arrest, this should be further evaluated. siRNA Rad51 or another HR factor may be a better way to impede DSB repair in G2.

We agree that there may be better perturbations than PARP inhibition and have chosen to remove that data. In Figure 7D, we now include siRNA to CtIP and inhibition of DNA-PK in addition to siRNA to RNF8. We find that these three treatments sustain H2B-ATKAR signal and impair mitotic entry after NCS addition.

We further tested siRNA to HR factors, including Rad51, as suggested by the reviewer. However, we fail to detect a significant effect on H2B-ATKAR after transfection of these siRNAs. We have therefore reformulated the paragraph to stress that the model and predictions concerns DNA damage that is sensed by ATM.

We apologize that the description for modeling during sustained damage was lacking. We have now included a description in materials and methods and run the model to steady state for a range of sustained damages to better detect a threshold (Figure 7C).

Referee #2

(Report for Author)
In this study Jaiswal et al., constructed two reporters in order to monitor the activity of DNA damage response (DDR) in living cells. They show that the generated reporters, although initially constructed to report ATM- and ATR-mediated activity, they specifically respond to ATM activation. In order to be able to monitor ATM activity at different subcellular locations, they constructed one chromatin bound and one diffusible reporter. These reporters showed different kinetics of ATM activity upon NCS treatment, but surprisingly the authors didn't correlate their response with the kinetics of known targets of ATM upon NCS (see below). By using a similar reporter that probes Plk1 activity, they correlated Plk1 activation with dephosphorylation of the chromatin bound reporter upon NCS treatment. Using the chromatin bound ATM reporter the authors showed that, upon NCS or localized DNA damage, ATM activity is detected throughput the chromatin. Surprisingly however they didn't detect an enrichment of ATM activation at DNA damage foci or at laser-induced damage sites, where, clearly, known targets of ATM (including ATM autophosphorylation, Figure 6D, or pSMC3, SupplFigure 8A) are enriched. They nicely showed that Wip1 phosphatase can counteract ATM activity on chromatin and they assembled a mathematical model that allowed to predict that ATM activity on chromatin enforces a checkpoint arrest above a threshold of damage and ensures a minimal duration of the checkpoint.
In general the study aims at understanding of how cell cycle could restart before completion of DNA damage, an interesting topic that has implications for understanding the mechanism of checkpoint adaptation in human cells. Although the findings presented in this manuscript are interesting, it is difficult to assess the quality of the observations as statistics are missing across the study. In addition the characterization of the main tools of the study, the two reporters, is rather weak (see below) and the effect of ATM activity on the duration of the cell cycle is rather correlative. Assessing the criticism raised as major and minor points below will substantially improve the manuscript, before consideration for publication in EMBOJ.

We thank the reviewer for the constructive comments.

Major comments:

I would expect an early in the study direct comparison of the kinetics of targets of ATM upon damage (e.g NCS or laser induced damage) such as Kap1-S824, SMC-S1083 or ATM autophosphorylation with the kinetics of both the immobilized onto chromatin or the soluble ATKAR reporter. This is a crucial experiment which will help the authors decide which of the two reporters can be used to accurately follow ATM activity in live cells. This is an important point since: 1) the reporters show different responses upon NCS treatment and 2) the authors could not detect an enrichment of H2B-ATKAR activity on DNA damage foci (while both gH2AX and pS1981 ATM staining persisted), suggesting that the chromatin bound reporter actually does not report the ATM-dependent phosphorylation events that take place onto chromatin (e.g the early DDR activation events). Similarly, signal from the same reporter started to revert within an hour after the NCS addition, which, as the authors mentioned, suggests that "the ATM-mediated phosphorylation of an artificial substrate becomes less efficient when the substrate is targeted to chromatin". However, in most of the experiments in the study the same chromatin-bound reporter was used. I would suggest that the authors perform a direct comparison of the activity of their reporters and known ATM targets on chromatin and nucleoplasm.

We completely understand the reviewer's point that phosphorylation of endogenous targets could be introduced early in the manuscript. In fact, that was how initial versions of the manuscript were written. However, we believe that readability and clarity increased by first describing the probes and later compare to endogenous ATM targets.

In new figure EV4A we now show fractionation and WB for endogenous ATM targets at different times after NCS addition. These new data supplement the existing figure 8A, in which we show that various endogenous ATM targets are dephosphorylated with different dynamics. We provide an explanation for this phenomenon by showing that an artificial ATM target is dephosphorylated differently depending on its intranuclear localization. We show examples of ATM targets (ATM p1981, gH2AX, CHK2-pT68, and as comparison CHK1-S317) that similar to ATKAR are retained long after DNA damage. In contrast, KAP1-S824 and p53-pS15 are similarly to H2B-ATKAR rapidly dephosphorylated before Plk1 activity resumes. Further, endogenous targets show different localization: whereas gH2AX largely remains at DDR foci, pKAP1 is localized throughout chromatin, pCHK2 and pCHK1 are mostly soluble and many other proteins can likely shuttle between both compartments. Thus, it is in our opinion not meaningful to say that one of the probes is more accurate than the other. Endogenous ATM targets follow different dynamics and we here provide a mechanism that at least partly can explain this phenomenon. Whereas PP4 resides mostly in soluble fraction, WIP1 is present exclusively at chromatin suggesting that the level of the target phosphorylation may be controlled not only by the activity of the upstream kinase but also by the access to its phosphatase.

We do not detect H2B-ATKAR phosphorylation at DDR foci, which could be due to several reasons. First, the amount of H2B-ATKAR may be low on DDR focus compared to H2B-ATKAR on surrounding chromatin in same pixel. In this sense, detection of a ratio-change is less efficient than detection of enrichment of a tagged protein whereas the technical imaging challenges are higher. In addition, during resection or remodeling, the amount of nucleosomes in DDR foci containing H2B-ATKAR may be reduced. Further, despite significant efforts from several labs, including ours, no one has to our knowledge succeeded in detecting FRET-change on a probe targeted to the centrosome. The reason for this is unknown, but it remains a possibility that FRET-probes may be restricted to change conformation in the dense cross-linked protein environment surrounding the centrosome. It is possible that DDR foci contain similar protein environments, thus making FRET changes difficult to study there. Nonetheless, we show that outside DDR foci the ATM responsive H2B-ATKAR follows similar desphosphorylation dynamics as the endogenous ATM target pKAP1. As H2B-ATKAR dephosphorylation, but not ATKAR dephosphorylation, correlated to when Plk1 activity resumed, we in this manuscript focused mainly on studying H2B-ATKAR.

I was also rather disappointed to see that across the manuscript there was not a single experiment with the appropriate statistics. Especially in live experiments where the number of the reported analyzed cells was 10-20, it is very difficult to estimate the significance of the findings.

We have now introduced statistical tests to Figure 4. However, we are afraid that applying

statistical tests for live-cell data showing multiple time-points and conditions may complicate the figures and reduce readability. We therefore decided to adhere to the editor's comment that dedicated statistical tests may only be needed for panels where the presentation is not by itself already intuitively meaningful.

Minor:

Although the main conclusions of the study are drawn based on the ATM/ATR Activity reporters the authors haven't explained how these reporter's actually work. Including a scheme of the reporters would help readers not familiar with the FRET-based methodologies to understand how these reporters in principle report ATM-dependent phosphorylation.

We agree and have now included a scheme as new Figure 1A.

How the reporters respond when cells are pretreated with ATMi inhibitor before the induced DNA damage (e.g laser-induced, NCS).

These data were already present in the manuscript (Figures 1A (now Figure 1B) and S1E (now EV1E) for NCS, and figure 5C for laser-microirradiation)

Referee #3

(Report for Author)
Comment on the modeling part of the manuscript "ATM/Wip1 activities at chromatin control Plk1 re-activation to determine G2 checkpoint duration [EMBOJ-2016-96082]"

In this manuscript, the authors study the cell cycle response to DNA damage. DNA damage activated the kinase ATM, which phosphorylate their targets to block cell cycle progression. On top of this, the counter acting phosphatase Wip1 dephosphorylate the target proteins of ATM. Hence, the target proteins could be de-phosphorylated and re-activated before the complete inactivation of the kinases ATM. As a result, the cell cycle progression is able to continue in the presence of residual DNA damage and ATM activities. Overall, this is a logical story that makes intuitive sense.

We thank the reviewer for the constructive comments.

In the manuscript, the authors also incorporated a simple mathematical model. The current model has some flaws:

One: the current model is oversimplified and does not take into consideration of the biological details of the DNA damage checkpoint. Given that detailed mathematical models have been constructed on the DNA damage checkpoint, it is better to use and modify these detailed models rather than constructing an over simplified version.

We respectfully disagree. The model we devised is not intended to capture every detail, but due to the low number of parameters, to robustly show insight into a novel mechanism concerning how recovery from a G2 checkpoint is regulated.

As reviewer one commented: "This represents a complex but interesting paper and, importantly, provides evidence for a novel mechanism for the regulation of mitotic entry after DSB induction". We believe that the model we devised both functions as a framework to explain our findings and as a mean to both verify and predict the general consequences of our experimental findings.

We do agree that a detailed model would allow additional analyses. However, to be accurate, such a model would preferably incorporate DDR foci, chromatin, nucleoplasm and cytoplasm, and a large number of constants that currently are unknown would need to be determined. It is our long-term goal to assemble a data-driven functional model with spatial constraints, and we hope that the insights, tools, and data we developed in this study will form a base for such efforts.

Two: the model parameters should be constrained by biological data, and the model simulation should be compared to the experimental observations so that the model can be integrated to the

experimental work in this manuscript and the data from the literature.

In new Figure EV3A-G we now use biological data to constrain parameters and to compare model results to experimental data.

Three: the model prediction, that there is a threshold of DNA damage, should be properly analyzed with nonlinear tools such as one parameter bifurcation analysis. Furthermore, whether such a threshold functions in the control of cell cycle checkpoint should be tested within the experimental framework proposed in this manuscript.

We thank the reviewer for the suggestion. In new Figure 7C we now run the model to steady state for a range of sustained damages, indicating the position of the threshold. In Figure 7D, we now show that cells treated with CtIP or RNF8 siRNA, or DNA-PK inhibitor sustain H2B-ATKAR phosphorylation and fail to enter mitosis after NCS addition.

In conclusion, the reviewer suggests that the current mathematical model should be greatly improved following the above suggestions. The end result might then be an impactful manuscript combining experimental and theoretical work that is suitable for the publication on EMBOJ.

2nd Editorial Decision 03 May 2017

Thank you for submitting your revised manuscript on ATM/Wip1 control of checkpoint duration. We have now carefully considered your responses, and the manuscript has also been re-reviewed by one of the original referees (see comments below). As a result of these assessments, I am happy to inform you that we see no further objections towards publication in The EMBO Journal, except for a few remaining editorial issues that need to be addressed.

REFEREE REPORT

Referee #1

(Report for Author)
I have gone through this revision and the authors have addressed all my comments and I am now happy to recommend acceptance. I stress that I cannot efficiently assess the modeling aspect of the paper. I have ticked the box of medium significance - I find the paper significant and important but it is very complex and difficult to critically evaluate, which dampened my enthusiasm for it being of high significance. The mechanistic insight gained from the work is high though.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Arne Lindqvist, Libor Macurek
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2016-96082

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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B- Statistics and general methods

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1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size limited by experiments
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C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Antibodies and corresponding catalogue numbers are described in materials and methods
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cells were regularly tested for mycoplasma, information on cell types in materials and methods

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA

14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	No data availability section included
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	A SBML version of the model is submitted to biomodels and the accession referenced in materials and methods

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA