THE EMBO JOURNAL

Manuscript EMBO-2010-73661

14-3-3γ mediates Cdc25A proteolysis to block premature mitotic entry after DNA damage

Kousuke Kasahara, Hidemasa Goto, Masato Enomoto, Yasuko Tomono, Tohru Kiyono, Masaki Inagaki

Corresponding author: Masaki Inagaki, Aichi Cancer Center Research Institute

Review timeline:

Submission date:	14 January 2010
Editorial Decision:	15 February 2010
Revision received:	21 April 2010
Editorial Decision:	25 May 2010
Revision received:	18 June 2010
Accepted:	21 June 2010

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 15 February 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three expert reviewers, whose comments are copied below. As you will see, they all appreciate the amount and quality of experimental data and also consider your new findings on the mechanism of Chk1-dependent Cdc25 & cell cycle regulation interesting and potentially important. They nevertheless raise a number of specific issues regarding experiments, presentation, and interpretation of the results, which would nee to be adequately addressed prior to publication. A major concern in this respect is that some of the conclusions are currently not supported by sufficiently strong evidence to decisively rule out possible alternative interpretations.

Should you be able to satisfactorily respond to these various points, we should be able to consider publication of a manuscript revised along the lines suggested by the reviewers. Please be reminded that it is EMBO Journal policy to allow a single round of major revision only, and that it will thus be important to diligently answer to all the various experimental and editorial points raised at this stage. Please, also make sure to carefully edit and proofread the study, ideally involving a native speaker of English in this case. Finally, when preparing your letter of response, please also bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html). In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

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

Yours sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Although many laboratories have contributed to our understanding of Chk1 regulation by ATR, there are still many holes in our knowledge of how this essential kinase is regulated by DNA damage and the factors that mediate interaction and regulation of its substrates. The model from several laboratories is that the C terminus of Chk1 contains domains involved in both activation and inhibition of t he kinase. Phosphorylation by ATR on the C-terminus could result in a conformational change that exposes the catalytic domain to its substrates. Further phosphorylation in a Chk1-dependent manner, presumably by auto-phosphorylation may stabilize the "active conformation of the kinase"; at the same time exposing the kinase to phosphatases that will inactive the kinase during recovery from the DNA damage checkpoint. Furthermore, this is still a working model, one limiting factor to our knowledge is the small number of interacting proteins and substrates that are known for Chk1. In this manuscript Kasahara, et al., use a known substrate of Chk1 to understand how post-translational regulation of Chk1 and protein-protein interactions may regulate checkpoint signaling following DNA damage. Kasahara et al., investigate whether phosphorylation of Chk1 at Ser296 and interaction with a 14-3-3 isoform could provide a platform for interaction and phosphorylation of a critical substrate of Chk1, the phosphatase Cdc25A Phosphorylation of Cdc25A by Chk1 and other kinases has been shown to be necessary for its degradation to achieve delay of S phase and mitosis following DNA damage.

The generous amount of data that are of very high quality support a model in which Chk1 is phosphorylated on Ser296 following DNA damage, which is necessary for both interaction with 14-3-3 and phosphorylation of Cdc25 on critical residues required for its ubiquitylation. Although the data are of high quality the authors fall short of showing that the phosphorylation of Chk1 on Ser296 is due to auto-phosphorylation and that 14-3-3 sigma is providing a platform for interaction with Cdc25A in a purified system.

Major concerns.
Results page 7-8 The authors do not provide any evidence for the following statement:

This observation is consistent with a previous report that protein phosphatase 2A
8 (PP2A) promptly dephosphorylates ATR sites in a Chk1 kinase activity-dependent
manner (Leung-Pineda et al, 2006). Thus, Ser296 phosphorylation, which occurs only on
Chk1 phosphorylated at ATR sites, is likely to promote rapid dephosphorylation at ATR
sites by protein phosphatases such as PP2A (Leung-Pineda et al, 2006).

The authors show that phosphorylation at Ser296 is dependent on ATR phosphorylation sites in vivo. They use recombinant protein isolated from insect cells to show that all three sites (ATR + Ser 296) are phosphorylated in insect cells. They further show that upon incubation with phosphatase they can reverse phosphorylation at all sites and addition of ATP under phosphorylation-permissive conditions restores phosphorylation only on Ser296. The authors conclude that these data definitively show that phosphorylation at Ser296 is due to auto-phosphorylation. Although this is likely to be the case, the data presented does not conclusively show this, and poses the following problems. If the ATR sites are necessary for phosphorylation of Ser296 in vivo, the authors must explain why phosphorylation at these sites is no longer required in vitro. My second concern is that Chk1 expressed in insect cells co-precipitates with insect cell-associated proteins and it is likely that a contaminating kinase is present in those preps. To make the argument that no eukaryotic kinase phosphorylates Chk1 at Ser296 they need to show that Chk1 kinase expressed in bacteria an auto-phosphorylate on Ser296.

Supp Fig. 3B. Insect cell GST Chk1 S296A when incubated in the presence of ATP still shifts just like WT protein, yet the kinase defective mutated protein does not shift. How many Chk1 kinase activity-dependent sites are there and why is Ser296 the only critical auto-phosphorylation site?

Figure 5D: UCNO1 treatment during UV radiation reduced binding of Chk1 to 14-3-3 gamma. However, UCN01 had no effect on the destabilization of Cdc25A following damage, suggesting that in this assay UCN01 did not inhibit Chk1, can the authors reconcile these results?

The experiments in figure 6 are not explained in detail.
6A 1) Is the expression of Chk1Ser296A blocking Cdc25A ubiquitination even in the presence of the endogenous protein?
2) In addition, there was no ubiquitin modified forms of Cdc25A observed in the last lane of Figure 6A suggesting that this was only observed in cells overexpressing Chk1 (read below).

6B The experiments showing that 14-3-3 depletion impacted the levels of ubiquitin conjugated Flag-tagged Cdc25A were carried out in cells overepxressing WT-Myc tagged Chk1. Was transfection of Myc-tagged wild type Chk1 necessary for ubiquitination of Flag-tagged Cdc25A seen in 6B?'

The in vitro kinase reactions to measure the impact of Ser296A mutation in kinase activity of Chk1 were carried out under different conditions using Cdc25C (Fig. 3A) and or Cdc25 A (Fig. 6 C and D) as substrate.

Interpretation of results. Except for the experiments in figure 6C there could be alternative explanation for these findings. Since Cdc25A regulation occurs when the nuclear membrane is intact. A possible explanation for these results is that Chk1S296A or cells lacking 14-3-3 gamma fail to localize Chk1 to the appropriate cellular compartment so that it can interact with Cdc25A. Although the IP's in figure 5F were carried out with total cellular extracts, this alternative explanation was not considered or ruled out.

Minor comments:
The citation for evidence that Chk1 is phosphorylated on Ser296 is not Palermo and Walworth (top of page 6).

Spelling of Histone H1 Figure 4 panel E (Histon H1)

Referee #2 (Remarks to the Author):

Review of EMBOJ-2010-73661 (Inagaki)

In this work, Inagaki and coworkers have presented evidence that phosphorylation of Chk1 on S296 promotes association with 14-3-3-gamma. This complex has a higher propensity to associate with Cdc25A. This association results in enhanced phosphorylation of Cdc25A on S76 by Chk1 and ensuing degradation of Cdc25A upon a checkpoint response to genotoxic stress. Overall, I believe that this manuscript presents thorough and convincing data in support of an interesting and significant hypothesis. Thus, I would support publication in the EMBO Journal pending minor revisions.

Points

1. Introduction and Discussion. The claim that 14-3-3 functions as an attachable NES in fission yeast has been discredited. Please remove these statements from the paper.

2. Figure 3D. There can be no phosphorylation by ATR in this in vitro assay, but phosphorylation of Chk1 on S296 occurs well, which contrasts with what happens in vivo. This discrepancy should be discussed.

3. Figure 4A. I am wondering why the authors did not add doxycycline earlier (before thymidine) so that the arrest could begin properly in cells containing normal Chk1.

4. Figure 4D. The quantitation should include all conditions (i.e., those involving the mock-treated cells).

5. Figure 4E. It should say "Histone H1."

6. Figure 5C. The authors should explain the experiment better in the text by saying that they have used siRNA treatment.

7. Figure 6. The experiments are poorly described in the legend.

Referee #3 (Remarks to the Author):

This is an interesting and comprehensive study showing that 14-3-3 gamma links Chk1 to Cdc25A degradation. The authors present strong evidence that auto-phosphorylation of Chk1 on serine 296 causes it to bind to 14-3-3 gamma and this subsequently promotes its interaction with Cdc25A, leading to enhanced degradation of Cdc25A. My one criticism is that the evidence for a ternary complex between Chk1, 14-3-3 gamma and Cdc25A is weak. The authors could strengthen their evidence either by expressing a dimerisation defective version of 14-3-3 gamma in their siRNA treated cells, which should not support the co-immunoprecipitation of Chk1 and Cdc25A after UV, or analyse the migration of the proteins on gel-filtration and show that Cdc25A, Chk1 and !4-3-3-gamma co-migrate.

Other points:
Fig. 2: The scale bars are said to be 20 micrometres but I find this hard to believe.
Fig. 4: Higher magnification images are required.

1st Revision - authors' response 21 April 2010

For Reviewer 1

1. ------the authors fall short of showing that the phosphorylation of Chk1 on Ser296 is due to auto-phosphorylation-----
(a) My second concern is that Chk1 expressed in insect cells co-precipitates with insect cell-associated proteins and it is likely that a contaminating kinase is present in those preps. To make the argument that no eukaryotic kinase phosphorylates Chk1 at Ser296 they need to show that Chk1 kinase expressed in bacteria an auto-phosphorylate on Ser296.
(b) Insect cell GST Chk1 S296A when incubated in the presence of ATP still shifts just like WT protein, yet the kinase defective mutated protein does not shift. How many Chk1 kinase activity-dependent sites are there and why is Ser296 the only critical auto-phosphorylation site?
(c) If the ATR sites are necessary for phosphorylation of Ser296 in vivo, the authors must explain why phosphorylation at these sites is no longer required in vitro.

The reviewer raised several concerns about our analyses using Chk1 protein purified from insect cells. According to the above suggestions, we added following new data.
(a) In order to rule out the possibility that a contaminating kinase in insect cells may phosphorylate Chk1-Ser296, we used His-ProS2-Chk1 protein expressed in bacteria according to the previous report with slight modifications (Zhao & Piwnica-Worms, Mol. Cell. Biol., 21, 4129-4139, 2001). By using such proteins, we obtained similar results that Ser296 phosphorylation occurred on WT but not on K38M or S296A (Figure 1C).
(b) We re-examined the in vitro kinase assays by using Chk1 purified from insect cells (Supplementary Figure S1E). Since no radioactive phosphate (32P) was incorporated into K38M protein, all site phosphorylations in vitro were dependent on Chk1 catalytic activity but not on contaminating kinase(s). Ser 296 mutation to Ala reduced 32P incorporation but not completely

abolished it. The 2D phosphopeptide mapping analyses also confirmed that Ser296 was one of the major autophosphorylation sites but (at most 4) other major phosphorylation sites existed. Since Ser296 phosphorylation was critical for 14-3-3 gamma binding, we focused on Ser296 phosphorylation in this paper. However, we canít rule out the possibility that other site phosphorylation(s) may also play critical roles in the functional change by Chk1 autophosphorylation. Therefore, we discussed this point carefully ("Discussion" section p19, line 14-18).
(c) The C-terminal regulatory domain of Chk1 was reported to inhibit its catalytic activation. ATR-induced phosphorylation is considered to cancel this inhibition in vivo (Katsuragi & Sagata, Mol. Biol. Cell, 15, 1680-1689, 2004; Walker et al., Oncogene, 28, 2314-2323, 2009). However, under the in vitro condition, the detergent treatment stimulated the catalytic activity in the absence of ATR-induced phosphorylation, likely through de-repression of C-terminal regulatory domain (Walker et al., 2009). We also obtained the similar results by using Chk1 protein expressed in bacteria. The catalytic activity toward Chk1-Ser296 was elevated by the usage of 1% sarcosyl for bacteria lysis (Figure 1C), as reported (Zhao & Piwnica-Worms, Mol. Cell. Biol., 21, 4129-4139, 2001). In the case of Chk1 protein expressed in insect cells, we consider that the C-terminal inhibition is canceled during Chk1 purification. So, this protein is likely to maintain the catalytic activity without Chk1 phosphorylation at Ser317 and Ser345. We discussed this point carefully ("Discussion" section p18 line 16-p19 line 9).

2. ------the authors fall short of showing --- that 14-3-3 gamma is providing a platform for interaction with Cdc25A in a purified system.

According to the suggestion, we performed an in vitro binding assay by using purified proteins. 14-3-3 gamma enhanced the interaction between Cdc25A and Chk1 in a purified system (Figure 5H).

3. The authors do not provide any evidence for the following statement: This observation is consistent with a previous report that protein phosphatase 2A (PP2A) promptly dephosphorylates ATR sites in a Chk1 kinase activity-dependent manner----------.

According to the suggestion, we added new data to demonstrate the above. The level of dephosphorylation at Ser317 and Ser345 after the release of hydroxyurea was drastically decreased by the treatment with UCN-01 or the depletion of PP2A (Supplemental Figure S2C and S2D).

4. UCNO1 treatment during UV radiation reduced binding of Chk1 to 14-3-3 gamma. However, UCN01 had no effect on the destabilization of Cdc25A following damage, suggesting that in this assay UCN01 did not inhibit Chk1, can the authors reconcile these results?

The reviewer raised a question about the effect of UCN-01 toward Cdc25A stability. In a previous manuscript, we performed the experiment in the presence of MG132, a proteasome inhibitor. So, MG132 treatment attenuated the effect of UCN-01 toward Cdc25A stability. However, we agreed with your opinion that the above experiment may confuse the readers. So, we performed the experiment in the absence of MG132. UCN-01 treatment not only stabilized Cdc25A but also attenuated 14-3-3 gamma binding to Chk1 and Cdc25A (Figure 5D).

5. The experiments in figure 6 are not explained in detail.
6A 1) Is the expression of Chk1Ser296A blocking Cdc25A ubiquitination even in the presence of the endogenous protein?
2) In addition, there was no ubiquitin modified forms of Cdc25A observed in the last lane of Figure 6A suggesting that this was only observed in cells overexpressing Chk1 (read below).
6B The experiments showing that 14-3-3 depletion impacted the levels of ubiquitin conjugated Flag-tagged Cdc25A were carried out in cells overepxressing WT-Myc tagged Chk1. Was transfection of Myc-tagged wild type Chk1 necessary for ubiquitination of Flag-tagged Cdc25A seen in 6B?

(a) The reviewer raised a question about the effect of endogenous Chk1 in Chk1-overexpressing cells. We also performed the experiment by using endogenous Chk1-depleted cells. Since similar results were obtained in the case of endogenous Chk1 depletion (Figure 6A), endogenous Chk1 appeared to have little effects on Cdc25A polyubiquitylation in this experimental system.
(b) The reviewer raised a concern that the reduction in Cdc25A polyubiquitylation by 14-3-3 gamma

depletion might be observed only in Chk1 WT-overexpressing cells. We performed the experiment without Chk1 overexpression. 14-3-3 gamma depletion attenuates Cdc25A polyubiquitylation even without the induction of exogenous Chk1 (Figure 6C).

6. The in vitro kinase reactions to measure the impact of Ser296A mutation in kinase activity of Chk1 were carried out under different conditions using Cdc25C (Fig. 3A) and or Cdc25 A (Fig. 6 C and D) as substrate.

According to the suggestion, we also used Cdc25C as a substrate in the in vitro kinase assay (Figure 6F). Like Cdc25A-Thr507 phosphorylation, Cdc25C-Ser216 phosphorylation depended on the amount of Chk1 protein rather than that of 14-3-3 gamma. So, the requirement of 14-3-3 gamma is relatively specific to Cdc25A-Ser76 phosphorylation.

7. Interpretation of results. Except for the experiments in figure 6C there could be alternative explanation for these findings. Since Cdc25A regulation occurs when the nuclear membrane is intact. A possible explanation for these results is that Chk1S296A or cells lacking 14-3-3 gamma fail to localize Chk1 to the appropriate cellular compartment so that it can interact with Cdc25A. Although the IP's in figure 5F were carried out with total cellular extracts, this alternative explanation was not considered or ruled out.

According to the suggestion, we examined the subcellular localization of Myc-Chk1 WT or S296A before or after UV irradiation. We observed only marginal changes in the nucleus/cytoplasm ratio between 2 proteins (data not shown). In addition, 14-3-3 depletion had few impacts on Chk1 localization before or after UV irradiation (data not shown). So, nuclear accumulation of Chk1 is likely to be regulated by Chk1-Ser345 phosphorylation (Jiang et al., J. Biol. Chem., 278, 25207-25217, 2003) rather than Chk1-Ser296 autophosphorylation and 14-3-3 association. We discussed this point carefully ("Discussion" section p17 line 15-p18 line 2).

8. The citation for evidence that Chk1 is phosphorylated on Ser296 is not Palermo and Walworth (top of page 6).

According to the suggestion, we cited another journal (Clarke & Clarke, Biochem. J., 388, 705-712, 2005).

9. Spelling of Histone H1 Figure 4 panel E (Histon H1)

We corrected the spelling. Thank you.

For Reviewer 2

1. Introduction and Discussion. The claim that 14-3-3 functions as an attachable NES in fission yeast has been discredited. Please remove these statements from the paper.

According to the suggestion, we completely removed the statements that 14-3-3 functions as an attachable NES in fission yeast.

2. There can be no phosphorylation by ATR in this in vitro assay, but phosphorylation of Chk1 on S296 occurs well, which contrasts with what happens in vivo. This discrepancy should be discussed.

The C-terminal regulatory domain of Chk1 was reported to inhibit its catalytic activation. ATR-induced phosphorylation is considered to cancel this inhibition in vivo (Katsuragi & Sagata, Mol. Biol. Cell, 15, 1680-1689, 2004; Walker et al., Oncogene, 28, 2314-2323, 2009). However, under the in vitro condition, the detergent treatment stimulated the catalytic activity in the absence of ATR-induced phosphorylation, likely through de-repression of C-terminal regulatory domain (Walker et al., 2009). We also obtained the similar results by using Chk1 protein expressed in bacteria. The catalytic activity toward Chk1-Ser296 was elevated by the usage of 1% sarcosyl for bacteria lysis (Figure 1C), as reported (Zhao & Piwnica-Worms, Mol. Cell. Biol., 21, 4129-4139, 2001). In the case of Chk1 protein expressed in insect cells, we consider that the C-terminal

inhibition is canceled during Chk1 purification. So, this protein is likely to maintain the catalytic activity without Chk1 phosphorylation at Ser317 and Ser345. We discussed this point carefully ("Discussion" section p18 line 16-p19 line 9).

3. I am wondering why the authors did not add doxycycline earlier (before thymidine) so that the arrest could begin properly in cells containing normal Chk1.

According to the suggestion, we also performed the experiments by adding doxycycline at 1 day before thymidine addition (Supplementary Figure S4). We obtained results similar to the data shown in Figure 4.

4. The quantitation should include all conditions (i.e., those involving the mock-treated cells; Figure 4D).

According to the suggestion, we presented the quantification data containing all conditions (Figure 4D).

5. It should say "Histone H1."(Figure 4E)

We corrected the spelling in Figure 4E. Thank you.

6. The authors should explain the experiment better in the text by saying that they have used siRNA treatment (Figure 5C).

According to the suggestion, we explained Figure 5C experiment in the text more precisely ("Result" section, p12, line 13-15).

7. The experiments are poorly described in the legend (Figure 6).

According to the suggestion, we explained Figure 6 experiment not only in the legend but also in the text ("Result" section, p13, line 20-p15, line 7) more precisely.

For Reviewer 3

1. My one criticism is that the evidence for a ternary complex between Chk1, 14-3-3 gamma and Cdc25A is weak. The authors could strengthen their evidence either by expressing a dimerisation defective version of 14-3-3 gamma in their siRNA treated cells, which should not support the co-immunoprecipitation of Chk1 and Cdc25A after UV, or -----.

According to the suggestion, we replaced endogenous 14-3-3 gamma with GFP-14-3-3 gamma WT or a dimerisation defective mutant (DM; Supplementary Figure S5). Compared with WT case, the replacement with GFP-14-3-3 gamma DM abolished the complex formation between Chk1 and Cdc25A although there were only marginal change in the interaction between Chk1 and GFP-14-3-3 gamma (Figure 5G). So, the complex formation between Chk1 and Cdc25A is required for the dimerization of 14-3-3 gamma.

2. The scale bars are said to be 20 micrometres but I find this hard to believe (Figure 2).

We mistook 10 micrometers for 20 micrometers. We corrected the scale bar size in the legend of Figure 2. Thank you.

3. Higher magnification images are required (Figure 4).

According to the suggestion, we added higher magnification images of mitotic cells in Figure 4C.

2nd Editorial Decision 25 May 2010

Thank you for submitting your revised manuscript for our consideration. It has now been assessed once more by the original reviewer 1 (please see attached comments below), and I am happy to inform you that there are no more major objections towards publication in The EMBO Journal. As you will see, there remain however a few specific issues that would still need to be addressed. While I do not feel that this requires further experimentation, I would like to ask you to carefully respond and especially to address the remaining editorial issues, including a concern regarding the title of the paper. In addition, the manuscript would still strongly benefit from more careful and in-depth editing ideally by a native English-speaking scientists, not only to correct grammatical or language errors but also to improve readability in general by revising complicated and illogical phrases such as 'induce the decrease in polyubiqitylation'.

I am therefore returning the study to you once more, kindly inviting you to incorporate these additional changes into the manuscript text and to return the final version to us at your earliest convenience. After that, we should be able to swiftly proceed with its formal acceptance and production.

Yours sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Remaining Concerns:
1. Title

14-3-3 mediates Cdc25A proteolysis to induce S and G2
arrest after DNA damage

I may have missed it but I don't recall S phase checkpoint assays showing that cells expressing Chk1S296A failed in the S phase checkpoint.

2. I am still concerned that the effect that expression of Chk1 Ser296A has on Cdc25 phosphorylation and ubiquitylation after DNA damage is only examined using overexpressed proteins (Fig. 5).

3. Page 10 Figure 4D
I am surprised that Chk1 depletion by siRNA during a thymidine block did not result in massive cell death due to cells entering mitosis in the presence of unreplicated DNA (in non irradiated, vector transfected samples). This should have been reflected as a difference in mitotic index even after addition of nocodazole. I think authors should explain this.

4. Legend for Figure 6 remains confusing.

2nd Revision - authors' response 18 June 2010

Referee 1

1. Title "14-3-3 gamma mediates Cdc25A proteolysis to induce S and G2 arrest after DNA damage". I may have missed it but I don't recall S phase checkpoint assays showing that cells

expressing Chk1S296A failed in the S phase checkpoint.

As referee 1 pointed out, we did not clearly show data that cells expressing Chk1 S296A failed in the S phase checkpoint. So, we changed the title to "14-3-3 gamma mediates Cdc25A proteolysis to block premature mitotic entry after DNA damage".

2. I am still concerned that the effect that expression of Chk1 Ser296A has on Cdc25 phosphorylation and ubiquitylation after DNA damage is only examined using overexpressed proteins (Fig. 5).

Even using Myc-Chk1 WT-expressing Tet-ON cells, we could not detect endogenous Cdc25A phosphorylation and ubiquitylation after UV irradiation. It is likely due to technical limitations including lower sensitivity of antibodies to ubiquitin or phospho-Cdc25A, because several groups (Kang, T., Cancer Cell, 13 36-47 2008; Melixetian, M., et al. Nat. Cell Biol., 11, 1247-1253, 2009; Mailand, N., Science, 288, 1425-1429, 2000; Busino, L., Natutre, 426, 87-91, 2003; Jin, J., Genes Dev., 17, 3062-3074, 2003) also used exogenous Cdc25A for detection of Cdc25A ubiquitylation/phosphorylation.
However, we agree with the Refereeís opinion. In order to detect endogenous Cdc25A phosphorylation and ubiquitylation in cells, we are now trying to produce better antibodies. However, this will clearly take time. Thus, we wish to address the above issues in our future work.

3. Page 10 Figure 4D
I am surprised that Chk1 depletion by siRNA during a thymidine block did not result in massive cell death due to cells entering mitosis in the presence of unreplicated DNA (in non irradiated, vector transfected samples). This should have been reflected as a difference in mitotic index even after addition of nocodazole. I think authors should explain this.

In our experiments, we used Chk1 3íUTR siRNA, which has a milder effect on endogenous Chk1 reduction than the most commonly used siRNA (corresponding to nucleotides 127ñ147 of the human Chk1 coding region; Zhao, H., et al., Proc. Natl. Acad. Sci. U S A., 99, 14795-14800). Indeed, we could detect a higher level of endogenous Chk1 protein 24 h after transfection with Chk1 3íUTR siRNA than with the most commonly used siRNA. So, we consider that Chk1 remained to some extent during thymidine treatment (from 24 h to 40 h after siRNA transfection) and this was more than enough to arrest cells at the G1/S boundary. Around 48 h after siRNA transfection, we observed efficient reduction of endogenous Chk1 protein (Figure 4B). Thus, in our experimental system (Figure 4), we could evaluate the function of exogenous Chk1 protein in the DNA damage checkpoint. The above notion is also supported by the results in the case of exogenous Chk1 induction at an earlier time (please see Supplementary Figure S4).

4. Legend for Figure 6 remains confusing.

According to the suggestion, we added a schema showing Figure 6 (A-C) experiments in a new Supplementary Figure S6.

