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Proteolysis of Rad17 by Cdh1/APC regulates checkpoint termination and recovery from genotoxic stress

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

1st Editorial Decision 29 October 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. First of all I would like to apologise for the delay in getting back to you with a decision. Unfortunately, one of the referees was not able to return his/her report as quickly as initially expected.

Your manuscript has now been seen by three referees whose comments to the authors are shown below. As you will see while referee 1 is more negative the other two referees are more positive and would support publication here in principle if you could revise the manuscript in an adequate manner. Referee 1 is considerably more critical and feels strongly that in the absence of a considerably deeper mechanistic understanding of how the degradation of Rad17 is induced/regulated the paper would be better suited to publication in a more specialised journal. Now, I appreciate that asking for the ubiquitin ligase involved would presumably lie outside the scope of this study. Still, in the light these concerns I feel that it would be important to include at least some deeper insight into the regulation of Rad17 stability along the lines suggested. Taking together all these thoughts we will thus be able to consider a revised version of this manuscript if you can address the referees' concerns in an adequate manner and to their satisfaction.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For

more details on our Transparent Editorial Process initiative, please visit our website:
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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):

In this manuscript, Zhang et al describe the identification of Rad17 as a protein showing upregulation at early time points after damage, whereas the protein is degraded at later times. Rad17 protein turnover seems to be regulated by ubiquitin-dependent proteasomal degradation. The region of Rad17 required for degradation was identified and overexpression of a mutant version of Rad17 that lacks this degron region was shown to result in prolonged interaction of Rad9 with Claspin and prolonged Chk1 phosphorylation. Finally, elevated Rad17 protein levels were demonstrated in skin cancers.

Although the experiments are technically sound and well performed and the observations are potentially interesting for our understanding of DNA damage checkpoint regulation, the manuscript unfortunately lacks the mechanistic details of DNA damage-induced degradation of Rad17, such as what ubiquitin ligase is involved.

Major comments:
- With respect to mechanistic insights: Is the degradation dependent on ATR-mediated phosphorylation of Rad17?
- Apart from degradation of Rad17 at later time points after DNA damage, Rad17 levels also seems to go up at early time points. Is this 'stabilization' the effect of decreased turnover of Rad17 at such time points?
- The authors describe that UV exposure decreased the half-life of Rad17 from 8 to 4 hours, but the experiment in Figure 2A shows UV-treated cells, -/+ CHX. To investigate the effect of UV treatment on Rad17 stability, all samples should be treated with CHX, in the presence or absence of UV.
- Figure 2D: How long is MG132 treatment, same times as UV? Then increased Rad17 poly-ubiquitination upon time could be the result of accumulation of ubiquitinated Rad17 rather than stabilization upon DNA damage. All cells should be treated with MG132 for the same amount of time, for example during the last hour before harvesting the cells.
- I am not sure the 'scaffold model' for Rad17 that the authors describe for Rad17 function has been firmly proven in the literature. On the other hand, Rad17 has clearly been shown to regulate localization of Rad9 to the chromatin. I would therefore propose to study chromatin loading of Rad9 as a functional readout of Rad17 function (for WT versus Δ230-270) rather than Rad9-Claspin interaction.
- In the immunoprecipitation experiments only the proteins co-immunoprecipitating are shown. Controls should be shown to demonstrate the levels of the protein that is immunoprecipitated (Figure 3D: Rad9, Figure 3E: ATR, Figure 5A/B: Rad9, and Figure 6B: Rad9).
- The authors claim, and their results show, that overexpression of non-degradable Rad17 leads to a prolonged checkpoint. On the other hand, Rad17 seems to be upregulated in tumors, which theoretically would result in better checkpoint regulation. This contradictory needs to be explained.
- Although the observations in Figure 7 are interesting, the study is too limited to draw strong conclusions. It would be interesting to study the half-life of Rad17 in several tumor cell lines and correlate that to the level of Chk1 phosphorylation at later time points after the induction of damage.

Minor comments:
- Figure 3C: show DAPI and Rad17 in separate panels, to improve the visibility of Rad17 levels/foci.
- Figure 3B: the actual data points should be shown in the graph.
- Figure 4B: show expression of several Rad17 constructs also at t=0. In addition, the tubulin loading control is only from one set of samples and can be deleted unless a loading control for all samples is shown.
- Figure 7A: describe the origin of the cell lines used.
- The manuscript contains several grammatical errors and wrong references to figures (for example figure 2 on page 4 and figure 1 on page 9). Also the reference Francia et al 2006 on page 13 is wrong.

Referee #2 (Remarks to the Author):

Proteolysis of Rad17 regulates checkpoint termination and recovery from genotoxic stress

The findings presented by Zhang et al. are very important and yield novel insights into how genotoxin-activated checkpoint signaling pathways are regulated. The experiments are convincing and well-done, the results are presented logically, and the conclusions are supported by the data (with one major exception).

The following points are of concern, however.

1) On page 3 the authors state "In turn, phosphorylated Rad17 recruits the checkpoint complex 9-1-1 and loads it onto the lesion sites of damaged DNA. This process allows an association between the 9-1-1 complex and the ATR/ATM, and facilitates full activation of ATR/ATM, which triggers various downstream effectors such as Chk1, Chk2 and others to withdraw from cell cycle progression (Abraham, 2001; Cimprich and Cortez, 2008)." There are several concerns with these statements.

First, the statement "phosphorylated Rad17 recruits the checkpoint complex 9-1-1 and loads it onto the lesion sites of damaged DNA" is not supported by the models presented in Cimprich and Cortez, 2008, as well as multiple other primary articles and reviews. The current model is that the 9-1-1 complex is recruited independently of the ATRIP-ATR complex in mammals and S. cerevisiae. Thus, even though the authors have previously published that Rad17 phosphorylation is important for the interaction of Rad17 with the 9-1-1 complex, much additional data demonstrates that 9-1-1 loading onto chromatin is independent of ATR, which means that Rad17 phosphorylation cannot regulate the initial loading of the 9-1-1 complex. Instead, it is currently thought that the Rad17 phosphorylation is important for claspin recruitment (Wang et al, 2006, Mol. Cell 23:331-341).

Scond, the statement "This process allows an association between the 9-1-1 complex and the ATR/ATM, and facilitates full activation of ATR/ATM, which triggers various downstream effectors such as Chk1, Chk2" is also not entirely correct. The 9-1-1 complex does not directly activate ATR. Instead, TopBP1 is the critical intermediary. In addition, there is scant evidence that the 9-1-1 complex activates ATM and Chk2. In fact, careful analyses of Rad9 and Hus1 and Rad17 knockout cells have repeatedly shown that the 9-1-1 complex is not required for Chk2 activation.

2) The authors have an entire section entitled "Impaired Rad17 proteolysis is involved in carcinogenesis." While this is an interesting idea, no data are presented to support this bold contention. The only thing shown (which is not new) is that tumor-derived cell lines and patient biopsies have higher levels of Rad17. This increased level of Rad17 could be the result of many different mechanisms.

2) On page 6 the authors mention a special heat shock method for transfection. This must be fully described and the effect of this heat shock method on checkpoint signaling must be explored.

3) In Fig. 2C the authors show that MG-132 causes the stabilization of Rad17. Since MG-132 works by blocking the proteolysis of polyubiquitinated proteins, we should see that MG-132 leads to the accumulation of polyubiquitinated Rad17. This is clearly not what is seen.

4) In Fig 2D there are no controls for Rad17 ubiquitination. It is essential to show that in cells transfected with Myc-Ub (but without HA-Rad17) there are is no polyubiquitin smear. This is especially important since under the conditions shown, there are many polyubiquitinated proteins accumulating and many of these may stick nonspecifically to the immunoprecipitating beads. This might be especially important since the cells were transfected by a special heat shock method that could affect ubiquitination.

5) Need to show non-merged images in Fig. 3C. Also, it is unclear why no Rad17 is present at the zero time point.

6) On page 7 the authors state "These results suggest that in human primary cells, DNA damage enhances a transient association between Rad17 with checkpoint proteins such as Rad9 and ATR." The data, however, do not support this statement. What we see is an increase in the amount of Rad9 and ATR in the Rad17 immunoprecipitates. Because the levels of Rad17 must be increased in those samples (if the other figures in the paper are indeed correct), then if there must be more immunoprecipitated Rad17, so there will be more co-immunoprecipitated Rad9 and ATR. No change in the affinity of the interaction between Rad17 and the 9-1-1 complex is required for the results that are seen. The immunoprecipitates should be blotted to detect Rad17. Additionally, it is critical that the conclusion be revised.

7) Similarly, in Fig. 5 A-B the authors need to show total Rad17 present in immunoprecipitates.

8) Again in Fig. 6A-B the authors need to show total HA-Rad17 and the HA-Rad17 Δ 230-270 in the immunoprecipitates. Also, it is unclear to this reviewer how the authors specifically detected endogenous Rad17 [the band labeled Rad17 (endogenous) in Fig. 6A] since the cells contain both endogenous Rad17 and HA-Rad17 Δ 230-270, so both should show up when blotting for Rad17 (unless the anti-Rad17 Ab recognizes an epitope in the deleted region).

9) The authors claim that HA-Rad17 Δ 230-270 and endogenous Rad17 form dimers (misspelled as dimmers in the text). This data must be shown and they must discuss how they performed these experiments. It is not clear to this reviewer how they can perform these experiments.

Referee #3 (Remarks to the Author):

The DNA damage checkpoint pathway is activated in response to DNA damage or replication block, which arrests cell cycle to coordinate with DNA repair. While checkpoint activation has been extensively studied, mechanisms underlying the recovery from checkpoint following DNA repair is relatively less understood. Wang et al. showed that phosphorylation of Rad17 by ATR-ATRIP promoted claspin recruitment. Work from several labs indicated that ubiquitin-dependent degradation of Claspin by the SCF TrCP E3 ligase plays a critical role in terminating the checkpoint. The paper by Zhang et al. revealed that Rad17 is also subjected to ubiquitin-proteasomal degradation. Abrogation of Rad17 degradation prolonged Claspin association with ATR/9-1-1 complex and Chk1 activation, and delayed recovery from G2/M cell cycle arrest following UV exposure. This work is highly important in identifying Rad17 as a second component of the ATR/9-1-1/Rad17/Claspin checkpoint complex that is subjected to ubiquitin-proteosomal control following UV irradiation, and provided novel insight into the molecular mechanisms controlling cell cycle recovery from DNA damage checkpoint. The experiments are technically well performed and clearly presented. For these reasons, the manuscript is clearly worth publication in EMBO J after the authors address or discuss the following issues:

1. What is the relationship between Rad17 phosphorylation and the observed Rad17 degradation on chromatin? What triggers elevated Rad17 ubiquitination 4 hours after UV?

2. Rad17 Δ 230-270 inhibited Claspin degradation after UV, and prolonged Claspin association with 9-1-1 complex and Chk1 activation. Given that Claspin destruction by SCF TrCP is critical for checkpoint recovery, it would be beneficial to clarify the temporal relationship between Rad17 and Claspin degradation. Moreover, the phosphodegron is responsible for Claspin recognition by SCF TrCP. What is the phosphorylation status of Claspin in Rad17 Δ 230-270-expressing cells at

different times following UV treatment?

3. It is unclear whether Rad17 first dissociates from chromatin and is then ubiquitinated and degraded, or it is directly ubiquitinated on chromatin, which in turn facilitates its dissociation from chromatin and/or the checkpoint complex. The authors may want to comment this in the discussion.

4. The authors showed that Rad17 Δ 230-270 stabilized endogenous Rad17, and suggested that dimerization is a likely mechanism. This is an interesting finding and they may want to include the binding data.

5. In Fig. 6D, the percentage of G2/M cells at different time posts after UV should be indicated on the histograms.

1st Revision - authors' response 03 February 2010

Response to Reviewer #1

We agree with the reviewer that: "the experiments are technically sound and well performed and the observations are potentially interesting for our understanding of DNA damage checkpoint regulation." The major concern from the reviewer is that: "the manuscript unfortunately lacks the mechanism details of DNA damage-induced degradation of Rad17, such as the identity of the ubiquitin ligase involved."

To address this major concern, we have integrated into the revised manuscript our new finding that Cdh1/APC is the putative ubiquitin-protein ligase that governs UV-induced Rad17 proteolysis. We revised the abstract, added a new chapter reporting the identification/characterization of E3 ligase and modified the materials/methods, result (Figure 5A-H) and discussion sections appropriately. In general, we searched for UV-enhanced Rad17 interacting proteins by a TAP purification approach. Our findings that UV-irradiation promotes time-dependent interaction between Rad17 and Cdh1 and the subsequent molecular characterization of the effect of Cdh1 on regulating Rad17-mediated checkpoint response using RNA interference suggest that Cdh/APC is a putative E3 ligase that governs Rad17 proteolysis.

We have addressed each of the points raised by the reviewer and believe that we now have provided details of the mechanism by which Rad17 is regulated by the ubiquitin-proteasome system and clarified additional concerns raised by reviewer #1.

1) The reviewer asked whether "degradation is dependent on ATR-mediated phosphorylation of Rad17."

-At the time, we were fortunately able to demonstrate that UV-induced Rad17 is mediated by ATR but could not provide direct evidence to determine the necessity of Rad17 phosphorylation for its degradation due to unavailability of an assay system. As demonstrated in Supplemental Figure 1A & B, malfunction of ATR in GM18366 led to significant attenuation of UV-induced Rad17 oscillation and Chk1 phosphorylation suggesting that UV-induced Rad17 turnover is mediated by the checkpoint kinase ATR. The ideal assay system to measure whether phosphorylation of Rad17 is a prerequisite for its subsequent destruction would be an in vitro ubiquitylation assay of Rad17 with purified Cdh1/APC, E1 and E2. In such a system, S35 or P32 radialabeled Rad17 phosphorylated or unphosphorylated would serve as a substrate for ubiquitylation by purified Cdh1/APC, E1 and E2. While the results from our experiments using mass spectrometry/protein purification, co-immunoprecipitation, RNA interference and measurement of checkpoint response indicate that the E3 ligase role that catalyze the ubiquitylatoin of Rad17 is Cdh1/APC, we cannot presently examine the role of phosphorylation in Rad17 degradation due to lack of an appropriate assay system as discussed above.

2) The reviewer wanted us to address if the transient accumulation of Rad17 at the early time point after exposure of cell to UV is due to a decreased turnover of Rad17.

-Our new data based on our study of primary cultured human fibroblast cells suggest that Rad17 could be a constitutively turnover protein, and its time-dependent stabilization is required for its activation of checkpoint control. Our characterization of the interaction between Rad17 and Cdh1 by co-immunoprecipitation indicated a physiological association between Rad17 and Cdh1 in a static status, although enhanced interaction between Rad17 and Cdh1 was observed several hours later after UV treatment (Figure 5). We also observed that Cdh1 protein levels are tightly regulated in response to UV radiation with Cdh1 levels drastically dropping after exposure to UV, and this decline gradually reverses two hours after UV treatment. The correlative overlap of UV-induced oscillation of Rad17 and Cdh1 suggest that a decrease in the activity of Cdh1/APC in response to UV could result in a transient, stabilized Rad17 at the early time point.

3) The reviewer suggested a consistent design to measure Rad17 protein stability in both static status and condition after exposure to UV in the presence of cycloheximide.

- We redid the experiment following the reviewer's suggestion. As shown in Supplemental Figure 2, in the presence of cycloheximide, Rad17 protein levels dramatically dropped over the span of two hours after exposure to UV, while natural turnover (no exposure to UV) of Rad17 was observed after eight hours following treatment with cycloheximide.

4) The reviewer was curious about the treatment method using MG-132 and inquired whether MG-132 caused accumulation of Rad17 ubiquitin conjugates and whether the accumulation could be distinguished from the UV-induced Rad17 ubiquitylation.

-This is a good point. All dishes utilized in the experiments were pre-incubated with MG-132 one hour before exposure to UV radiation. Good number of previous reports indicated that MG-132 is a stable and long effective agent with effects lasting over 24 hours. Addition of MG-132 certainly enhanced the accumulation of Rad17 ubiquitin conjugates. It is technically difficulty to quantitatively distinguish the contribution of Rad17 ubiquitin conjugates between MG-132 and UV treatment. Nevertheless, the fluctuation of Rad17 ubiquitin conjugates after exposure cells to UV radiation in Figure 2D suggests the formation of Rad17 ubiquitin conjugates to UV response.

5) The reviewer challenged the term of "scaffold", which we used in the manuscript to describe a putative role of Rad17 associating with other checkpoint components such as 9-1-1 complex, as well as Claspin. The reviewer further suggests for us to perform additional experiments testing the chromatin loading of Rad9 by Rad17 (for WT versus Rad17 stable mutant).

-The role of Rad17 activated by ATR had been previously suggested to load 9-1-1 complex to the chromatin and incorporate Claspin for subsequently recruiting Chk1. Given the notion that Rad17 interact with checkpoint complexes as well as Claspin on the chromatin, we hypothesize its possible role as a "platform" providing a point of interaction for 9-1-1, ATR and Claspin on chromatin. As to the suggested experiment to test the role of Rad17 in loading Rad9 by using a non-degradable Rad17, the specific function of Rad17 in loading 9-1-1 to the chromatin is not a goal of the present work. This specific function was reported previously by various groups with inconsistent conclusions. The hypothesis that will be tested is whether regulation of Rad17 proteolysis could prolong the interaction of Claspin with the checkpoint complex, which would lead to an extended period of phosphorylation for Chk1. The proteolysis of Rad17 occurs over a span of four to six hours after exposure to UV. Based on the current paradigm, a possible role of Rad17 in loading 9-1-1 onto the chromatin would cease upon Rad17 proteolysis. Thus, a test to determine the effect of stabilized Rad17 on the interaction of Claspin and the checkpoint complex but not for loading of 9-1-1 is within the scope of the present hypothesis. An investigation of the controversial association between Rad17 and 9-1-1 is better suited for a future study. We hope the reviewer agree with our points.

Ideally, the best way to test the effect of wild-type and non-degradable Rad17 on loading 9-1-1 will be to generate Rad17 conditional knockout cell and then "rescue" the attenuated 9-1-1loading by over expressing wild-type or non-degradable Rad17, respectively. We obtained the conditional knockout cell from Dr. Lei Li at the MD Anderson Cancer Institute (Wang et al, 2003, Genes Dev).

Unfortunately, the poor health status of the cell in our hands wound not allow us to perform the suggested experiments.

6) Although we demonstrated the loading control for all immunoprecipitation experiments in the manuscript, we are suggested to show additional control indicating the levels of the protein that is immunoprecipitated such as Rad9 and ectopically expressed ATR.
-We have used Rad9 levels as an internal control to guide us in the equilibration of total protein utilized for the immunoprecipitation in Figure 3D, Figure 6A & B and Figure 7B. The predicated molecular mass for Rad9 in SDS-PAGE is approximately 50 KDa, which often overlaps with the location of IgG heavy chains. We attempted to determine this measurement in our pilot experiment, but it was unfortunately technically impossible in our hands. The predicted molecular mass for the tagged ATR is over 317 KDa, making it difficult to exhibit an entire IP-Western blotting in a regular format with the IgG bands and the super large tagged ATR. Thus, we measured the levels of ectopically expressed Flag-ATR in total lysate as a way to control for equal total protein used for the immunoprecipitation shown in Figure 7B.

7) We claim, and our results show, that overexpression of non-degradable Rad17 leads to a prolonged checkpoint. On the other hand, Rad17 seems to be upregulated in tumors, which theoretically would result in enhanced checkpoint regulation. This contradiction is asked to be explained.
-Although the aberrant Rad17 accumulation was observed in skin cancer/melanoma by us and in colon, breast and lung cancers by others, no direct evidence links UV-mediated Rad17 proteolysis to oncogenesis or tumor progression. Cancer takes time to develop. At this point, the aberrant Rad17 accumulation does not implicate it as a cause or consequence of cancer. We could only correlate the possible connection given the importance of Rad17 in mediating checkpoint control. We changed the overstatement in related paragraphs in the revised manuscript.

8) Although the observations in Figure 7 are interesting, the study is too limited to draw strong conclusions. It would be interesting to study the half-life of Rad17 in several tumor cell lines and correlate that to the level of Chk1 phosphorylation at later time points after the induction of damage.
-The purpose of the experiments shown in Figure 7 is only to provide indirect correlation between the molecular phenomena that UV-mediated Rad17 proteolysis with carcinogenesis but not to address the role of UV-mediated Rad17 proteolysis in the genesis of cancer. The suggestion for measuring half-life of Rad17 in several tumor cell lines and correlate that to the level of Chk1 phosphorylation at later time points after the induction of damage is out of the scope of the current manuscript and will be tested as part of future endeavor.

9) Figure 3C: show DAPI and Rad17 in separate panels, to improve the visibility of Rad17 levels/foci.
-Figure 3C is revised based on the suggestion.

10) Figure 3B: the actual data points should be shown in the graph.
-The relative fold value was added to Figure 3B.

11) Figure 4B: show expression of several Rad17 constructs at t=0. In addition, the tubulin loading control is only for one set of samples and can be deleted unless a loading control for all samples is shown.
- The tubulin loading control is removed following the suggestion from the reviewer. We did not include the data pertaining to t=0 because the aim of Figure 4B is to evaluate the protein stability of mutants over six to eight hours after exposure to UV radiation. The drop of protein levels for each of mutants at the eighth hour following UV exposure was evaluated as compared with its maximum expression levels during the time course.

12) Figure 7A: describe the origin of the cell lines used.
Information on the origin of cell lines has been included in the revised materials and methods.

13) The manuscript contains several grammatical errors and wrong references to figures (for example figure 2 on page 4 and figure 1 on page 9). Also the reference Francia et al 2006 on page 13 is wrong.

-All abbreviations of "Figure" inside of the parentheses in "Fig." in the manuscript have been changed to "Figure." The mistake in the reference for Francia et al 2006 on page 13 has been changed.

Response to Reviewer #2

We appreciate the reviewer's positive comments that: "*The findings presented by Zhang et al. are very important and yield novel insights into how genotoxin-activated checkpoint signaling pathways are regulated. The experiments are convincing and well-done, the results are presented logically, and the conclusions are supported by the data"*

We addressed each of the points raised by the reviewer as follows:

1) The review pointed out the caveats and concerns with the following statement in the introduction: "In turn, phosphorylated Rad17 recruits the checkpoint complex 9-1-1 and loads it onto the lesion sites of damaged DNA. This process allows an association between the 9-1-1 complex and the ATR/ATM, and facilitates full activation of ATR/ATM, which triggers various downstream effectors such as Chk1, Chk2 and others to withdraw from cell cycle progression (Abraham, 2001; Cimprich and Cortez, 2008)."
This model is based on cited review articles and some of recent findings.

First, the statement "phosphorylated Rad17 recruits the checkpoint complex 9-1-1 and loads it onto the lesion sites of damaged DNA" is not supported by the models presented in Cimprich and Cortez, 2008, as well as multiple other primary articles and reviews. The current model is that the 9-1-1 complex is recruited independently of the ATRIP-ATR complex in mammals and S. cerevisiae. Thus, even though the authors have previously published that Rad17 phosphorylation is important for the interaction of Rad17 with the 9-1-1 complex, much additional data demonstrate that 9-1-1 loading onto chromatin is independent of ATR, which means that Rad17 phosphorylation cannot regulate the initial loading of the 9-1-1 complex. Instead, the current thought is that Rad17 phosphorylation is important for claspin recruitment (Wang et al, 2006, Mol. Cell 23:331-341).

Second, the statement "This process allows an association between the 9-1-1 complex and the ATR/ATM, and facilitates full activation of ATR/ATM, which triggers various downstream effectors such as Chk1, Chk2" is also not entirely correct. The 9-1-1 complex does not directly activate ATR. Instead, TopBP1 is the critical intermediary. In addition, there is scant evidence that the 9-1-1 complex activates ATM and Chk2. In fact, careful analyses of Rad9 and Hus1 and Rad17 knockout cells have repeatedly shown that the 9-1-1 complex is not required for Chk2 activation.

-Following the critiques from the reviewer, we removed the cited literature from Cimprich and Cortex in 2008 and included additional references, which are consistent with the notion that supports the connection between Rad17 phosphorylation and loading of 9-1-1 complex. In addition, we incorporated the newly reported function of Rad17 in uploading Claspin onto chromatin that would then allow for the phosphorylation of Chk1 by ATR in the revised introduction with appropriate citations (Wang et al, 2006, Mol Cell 23:331-341). The role of Rad17 in loading 9-1-1 maintains controversial depending on cell type, genotoxic stress, and the period of experimental observation. While results from some group have not supported the "loading" concept based on their measurements at an early time point, increased Rad17/9-1-1 association was often found at DNA damage site in later time points.

Moreover, the statement "This process allows an association between the 9-1-1 complex and the ATR/ATM, and facilitates full activation of ATR/ATM, which triggers various downstream effectors such as Chk1, Chk2" is removed. Please read the revised whole paragraph in regards to the above two points criticized by the reviewer.

2) The authors have an entire section entitled "Impaired Rad17 proteolysis is involved in carcinogenesis." While this is an interesting idea, no data are presented to support this bold contention. The only thing shown (which is not new) is that tumor-derived cell lines and patient biopsies have higher levels of Rad17. This increased level of Rad17 could be the result of many different mechanisms.

-We agree with the reviewer's opinion that the statement was too broad without direct evidence linking the Rad17 proteolysis and carcinogenesis. We have limited the scope for all related statements in the revised manuscript.

3) The reviewer requested that we describe the enhanced transfection with gentle heat-shock and address the possible effect.

-We included the description in the revised materials and methods. The 40-second treatment at 42 C probably elicited only a brief response in the cell. The DNA damage checkpoint experiments were performed twenty four-hours after the transfection. Thus, the transfection should not adversely affect the designed experiments.

4) In Fig. 2C the authors have shown that MG-132 causes stabilization of Rad17. Since MG-132 works by blocking the proteolysis of polyubiquitinated proteins, we should see that MG-132 leads to the accumulation of polyubiquitinated Rad17. This is clearly not what is seen.

-In theory, blockage of proteasome function by the proteasomal inhibitor MG-132 results in an expected accumulation of Rad17 ubiuqitin conjugates. The explanation why such accumulation of Rad17 ubiquitin conjugates was not be tested in the experiments in Figure 2C is the following: (1) Formation of ubiquitin-conjugated substrate is technically difficult to be demonstrated by regular Western blotting because that modified substrate with ubiquitin forms smear, which decreases concentration of RAd17 ubiuqitin conjugates because the smeared migration is not sufficiently concentrated to be visualized by the limited sensitivity of antibodies and ECL; and (2) Some antibody efficiently picks up the antigen on the blot but its efficiency to detect ubiquitin-conjugated antigen may drop significantly.

5) In Fig 2D there are no controls for Rad17 ubiquitination. It is essential to show that in cells transfected with Myc-Ub (but without HA-Rad17) there are is no polyubiquitin smear. This is especially important since under the conditions shown, there are many polyubiquitinated proteins accumulating and many of these may stick nonspecifically to the immunoprecipitating beads. This might be especially important since the cells were transfected by a special heat shock method that could affect ubiquitination.

-This is a good point, we tested the possibility whether the beads used for the immunoprecipitation could co-pull down other epitopes that might possibly interfere with our experiment. Similar experiment as suggested was performed in our pilot studies. As shown in Supplemental Figure 3, the pilot result suggests that the myc-tagged and ubiquitin-conjugated Rad17 smear being detected in Figure 2D is signal specific.

6) Need to show non-merged images in Fig. 3C. Also, it is unclear why no Rad17 is present at the zero time point.
-We have modified Figure 3C according to the reviewer's suggestion.

7) On page 7 the authors state "These results suggest that in human primary cells, DNA damage enhances a transient association between Rad17 with checkpoint proteins such as Rad9 and ATR." The data, however, do not support this statement. What we see is an increase in the amount of Rad9 and ATR in the Rad17 immunoprecipitates. Because the levels of Rad17 must be increased in those samples (if the other figures in the paper are indeed correct), then if there must be more immunoprecipitated Rad17, so there will be more co-immunoprecipitated Rad9 and ATR. No change in the affinity of the interaction between Rad17 and the 9-1-1 complex is required for the results that are seen. The immunoprecipitates should be blotted to detect Rad17. Additionally, it is critical that the conclusion be revised.

-We agree with this comment. Results from our Western blotting suggested that the total protein abundance for Rad17 increased upon exposure to UV, while no significant changes occurred for Rad 9 and ATR. Thus, the alteration of immunocomplex of Rad9 and ATR as determined by co-immunoprecipitation of Rad17 is a result of Rad17 proteolysis. These results only reflect semi-

quantitatively dynamic interaction between Rad17 with Rad9 and ATR after exposure to UV but do not indicate their "association" (the term "association" could mislead reader to consider affinity, which is not supported by the present data). So, we adapted our conclusion in all related paragraphs.

8) Similarly, in Fig. 5 A-B the authors need to show total Rad17 present in immunoprecipitates.
-Same as our answer in the above (question 7), we agree with the reviewer's comment. Figure 1 A&B (endogenous Rad17) and Figure 4D (ectopically expressed Rad17) support the reviewer's comment that fluctuating profile of Rad17 interacting with Rad9 and ATR on the immuno-complexes in Figure 3&5 is due to UV-induced alteration of Rad17 and HA-Rad17. Obviously, the concentration of total Rad17 and HA-Rad17 that were co-pulled down on the immuno-complexes should be the same as that was observed for whole cell lysates as shown in Figure 1 A&B, Figure 4D and Figure 3&6. Thus, we modified the statement in the revised manuscript.

9) In Fig. 6AB the authors need to show total HA-Rad17 and HA-Rad17 Δ 230-270 in the immunoprecipitates. Also, it is unclear to this reviewer how the authors specifically detected endogenous Rad17 [the band labeled Rad17 (endogenous) in Fig. 6A] since the cells contain both endogenous Rad17 and HA-Rad17 Δ 230-270, so both should show up when blotting for Rad17 (unless the anti-Rad17 Ab recognizes an epitope in the deleted region).

- The experiment designed in the original Figure 6A is immunoblotting, not "imunoprecipitation". We have done a pilot experiment and found that antibody against Rad17 picks up both ectopically expressed wild-type and non-degradable Rad17. However, our previous effort could not provide a quality image that discloses both ectopically and endogenous Rad17 using the same antibody on one blot. So, we detected both ectopically expressed wild-type Rad17 and stabilized Rad17 with anti-HA antibody, and total Rad17, both ectopically and endogenously expressed, with anti-Rad17 using the same batch of whole cell lysates as a source from the same designed experiment. Per reviewer's request, we have tried several times to optimize our protocol. Now, we have obtained a reasonable image illustrating both ectopically expressed Rad17 (WT and stable mutant) with endogenous Rad17 on the same blot by using antibody against Rad17 (Figure 7A). The protein levels of endogenous Rad17 at time zero seems a little lower than what we have shown in other blots because of the difficulty of trying to show both endogenous and ectopically expressed Rad17. In addition, the status of total HA-Rad17 and HA-Rad17 230-270 in the immunoprecipitates with Rad9 in Figure 7B were reflected with the similarly designed experiments as shown in Figure 7A, which suggested that prolonged presence of Claspin with the checkpoint complex as mediated by Rad17 could be due to the stabilized Rad17 and is not related to the "affinity" or "association" that is argued by the reviewer #1 previously.

10) The authors claim that HA-Rad17 Δ 230-270 and endogenous Rad17 form dimmer. This data must be shown and they must discuss how they performed these experiments. It is not clear to this reviewer how they can perform these experiments.

-The conclusion that the non-degradable Rad17 could form "dimmer" with endogenous Rad17 is based on the result of the experiment that showed ectopically expressed/HA tagged Rad17 and endogenous Rad17 interacting together and co-present in the same immuno-complex (Supplemental Figure 4). At this point, we believe the statement " identification of dimmer based on our results" is not accurate. The interaction between non-degradable and endogenous Rad17 could be also due to possible formation of oligomers. This interaction between the ectopically expressed Rad17 with the endogenous Rad17 helps to explain the underline observation in Figure 7A that expression of stabilized Rad17 attenuate the turnover rate of endogenous Rad17 after exposure to UV. Thus, we changed the statement of "form dimmer" to "non-degradable and endogenous Rad17 interacting with each other."

Response to Reviewer #3

We appreciate the reviewer's positive comments: "*The experiments are technically well performed and clearly presented. For these reasons, the manuscript is clearly worth publication in EMBO J after the authors address or discuss the following issues."*

We addressed each of the points raised by the reviewer as following:

1) What is the relationship between Rad17 phosphorylation and the observed Rad17 degradation on chromatin? What triggers elevated Rad17 ubiquitination 4 hours after UV?
-Same question was raised earlier and was addressed in the reviewer#1-question 1. In the new chapter ñidentification of Cdh1/APC as a putative E3 ligase that governs UV ñ mediated Rad17 degradation, the results suggest that the time-dependent recovery of Cdh1 protein concentration is the mechanism that orchestrates the temporal proteolysis of Rad17. However, the detail for the triggering mechanism needs to be further studied.

2) Rad17 Δ 230-270 inhibited Claspin degradation after UV, and prolonged Claspin association with 9-1-1 complex and Chk1 activation. Given that Claspin destruction by SCFβTrCP is critical for checkpoint recovery, it would be beneficial to clarify the temporal relationship between Rad17 and Claspin degradation. Moreover, the phosphodegron is responsible for Claspin recognition by SCFβTrCP. What is the phosphorylation status of Claspin in Rad17 Δ 230-270-expressing cells at different times following UV treatment?

-This is a good question. In 2006, Michele Pagano and his group demonstrated that during hydroxyurea (HU) induced replication stress, the Plk1-phosphorylated Claspin is degraded by SCF/beta-TrCP mediated by a phosphodegron- DSGXXS. Destruction of Claspin contributes to termination of ATR-induced Chk1 phosphorylation thereby enabling cellular recovery from DNA replication stress. Similar findings were simultaneously reported by Jiri Lukas' group. Although similar molecular cascade including ATR, Rad17, Claspin and Chk1 is thought to mediate both UV-mediated DNA damage response and HU-induced DNA damage response, we are not sure if the potential mechanism for deactivating the checkpoint signaling would always require the multiple levels such as dephosphorylation of Chk1, degradation of Chk1 and destruction of Claspin. Further, to address the potential coordination between two ubiquitylation machineries (Cdh1/APC and SCF/beta-TrCP) in regulating the same signaling cascade at different layer is technically difficult. At this time, we could only speculate that probably both time dependent destruction of Rad17 and Claspin are involved in the checkpoint recovery. Stabilization of Rad17 somehow affects either the phosphorylation status of Claspin or the ubiquitylation of Claspin by the SCF/beta-TrCP, given the tight association between Rad17 and Claspin as demonstrated by several groups.

3) It is unclear whether Rad17 first dissociates from chromatin and is then ubiquitinated and degraded, or it is directly ubiquitinated on chromatin, which in turn facilitates its dissociation from chromatin and/or the checkpoint complex. The authors may want to comment on this in the discussion.

-This is a good question. Although this question is conceptually important, it is a technical challenge to address this issue directly. Although the result implies an association between the ubiquitylation of Rad17 and its destruction within the confine of the chromatin, we lack strong evidence on this point. The model of DDB-Cul4A ligase on regulating global genome nucleotide-excision repair via ubiquitylation of XPC and destruction of DDB2 as demonstrated by Tanaka K, Hanaoka F and Sugasawa K indirectly support our implication.

4) The authors showed that Rad17 Δ 230-270 stabilized endogenous Rad17, and suggested that dimerization is a likely mechanism. This is an interesting finding and they may want to include the binding data.
-Similar question was asked by the reviewer #2 (question 10). It has been addressed.

5) In Fig. 6D, the percentage of G2/M cells at different time posts after UV should be indicated on the histograms.
- The mean of percentage of G2/M cells at different time after exposure to UV was plotted in Figure 5E.

2nd Editorial Decision 17 February 2010

Thank you for sending us your revised manuscript. Our original referees 1 and 2 have now seen it again. In general, the referees are now positive about publication of your paper. However, both referees feel that there are a few issues that still need to be addressed (see below) before we can ultimately accept your manuscript. I would therefore like to ask you to deal with the issues raised. Given that both referees raise concerns regarding the tumor link put forward in figure 7 I would like to ask you to remove these data and the discussion referring to this issue.

Furthermore, there is one remaining editorial issue that needs further attention. Prior to acceptance of every paper we perform a final check for figures containing lanes of gels that are assembled from cropped lanes. While cropping and pasting may be considered acceptable practices in some cases (please see Rossner and Yamada, JCB 166, 11-15, 2004) there needs to be a proper indication in all cases where such processing has been performed according to our editorial policies. Please note that it is our standard procedure when images appear like they have been pasted together without proper indication (like a white space or a black line between) to ask for the original scans (for our records).

In the case of the present submission there is a panel that appears to not fully meet these requirements: figure 3E

I therefore like to kindly ask you to send us a new version of the manuscript that contains a suitably amended version of this figure. I feel that it would also be important to explain in the figure legend that all lanes come from the same gel. Please be reminded that according to our editorial policies we also need to see the original scan for the figure in question.

I am sorry to have to be insistent on this at this late stage. However, we feel that it is in your as well as in the interest of our readers to present high quality figures in the final print version of the paper.

Thank you very much for your cooperation. Please let us have a suitably amended manuscript as soon as possible.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

In the revised version of the manuscript by Zhang et al, the authors address the more mechanistic details of proteolysis of Rad17 and found Cdh1/APC ubiquitin ligase as an interactor of Rad17, suggesting that Cdh1/APC might regulate Rad17 levels in response to UV. Interestingly, Cdh1 levels are regulated in response to UV and the downregulation of Cdh1 by shRNA affects Rad17 stabilization and degradation (figure 5H, which should be quantified).
However, these experiments also raise additional questions. Given the fact that Cdh1 is strongly regulated throughout the cell cycle, one needs to determine whether the effect of Cdh1 downregulation on Rad17 levels is not merely a cell cycle effect. Alternatively, to strengthen the data that Cdh1 regulates Rad17 ubiquitination, the ubiquitination assay in figure 2D needs to be performed in the absence of Cdh1.

Although the manuscript significantly improved by the addition of these new data, I am disappointed by the way the authors addressed the majority of the other points I addressed. I would therefore strongly recommend that the following issues are (re)addressed before this paper is published in EMBO Journal:
1. A major issue was whether Rad17 degradation is dependent on ATR-mediated phosphorylation,

which is suggested by the experiments shown in supplemental figure 1. The authors argue that addressing this question is a difficult one, due to the lack of a appropriate assay, an in vitro ubiquitylation assay. However, this issue can easily be addressed by determining the effect of UV light on the proteins of a Rad17-S635A/S645A phosphorylation mutant as compared to wild type Rad17, in an experiment as performed in Figure 4D. This mutant was actually published by one of the authors (XF Wang) in an elegant Nature paper (Bao et al, 2001), so should be readily available to the lab.
2. Figure 4B: Rad17 degradation mutants are made and their stability upon UV damage are tested. I asked to demonstrate the levels of each mutant before the induction of DNA damage, but the authors argue that the aim of the experiment is to determine the protein stability in response to UV. The aim of the experiment is very clear to me but I think the control of the expression levels of these mutants before damage induction is essential to be able to draw any conclusions about these constructs, as it is well known that some (deletion) constructs do not express well/differently due to folding problems. The experiment in Figure 4B should therefore contain an additional lane showing levels of the Rad17 deletion constructs at t=0.
3. Throughout the manuscript many co-immunoprecipitation experiments are shown in which the authors show interactions of (for example) Rad17 with other proteins. Loading controls of input are shown but for me it is essential also to see the amount of protein that is immunoprecipitated, and not only the interacting protein (Figure 3D: Rad9, Figure 3E: ATR, Figure 5A/B: Rad9, and Figure 6B: Rad9). The authors argue that these experiments are difficult to do due to a number of reasons: Rad9 runs at the height of the heavy chain and ATR is too bit to see on the same gel. My suggestions for solving these problems: there are a number of good commercial anti-Rad9 antibodies of different species available. Perform the immunoprecipitation of Rad9 with a rabbit antibody, and the following western blot with a mouse of goat antibody. With respect to ATR: split the Flag-ATR IP sample into two, and run half on a gel to demonstrate Rad17 and the other half for immunoblotting for ATR or Flag.
4. The authors seem to have misunderstood my question about the graph of figure 3B. This graph now shows 2 smooth lines, which presumably fit the actual data points. I would like to see where in the lines the data points are situated, like has been done in figure 1C, 5F and 7E.
5. Finally, I am still uncertain whether the data describing the accumulation of Rad17 protein levels in tumour samples (Figure 7) is a valuable addition to this manuscript. To my suggestion to study the half-life of Rad17 in tumour cell lines the authors responded that this point is out of the scope of the current paper. I might agree with this but think that without such analysis, the performed data are too limited to draw a strong conclusion and would suit better in a separate manuscript discussing Rad17 levels in cancer in more detail.

Referee #2 (Remarks to the Author):

The author's have made reasonable efforts to address this reviewer's concerns. However, there are three concerns, including one major concern (#3).

1) In Point #4, reviewer 2 questioned why polyubiquitinated Rad17 did not accumulate in Fig. 2C. Although the authors responded, the response does not fit what is observed. Both of the provided rationales (smeared Rad17 due to ubiquitination and reduced immunoreactivity) would lead to lower levels of unmodified Rad17 in cells treated with MG132. That is not what is seen. Some explanation is required.

2) In Point #5, reviewer 2 requested that the authors show additional controls for Fig. 2D. The reviewer indicated that it was essential that the experiment contain a control in which the cells are transfected with Myc-Ub but no HA-Rad17. The author's response was to add Supplemental Fig. 3, but this figure does not show what was requested, thus leaving open the possibility the polyubiquitinated species that are observed may be due to the non-specific interactions of other cellular proteins that accumulate when cells are treated with MG132.

3) There remains a serious concern regarding the attempts of the authors to link their findings to carcinogenesis. There are three (at least occurances) First, they specifically mention a connection in the last sentence of the Summary. Second, the title of the relevant section in the Results is Impaired Rad17 Proteolysis Is Involved in Carcinogenesis. Third, there is a section entitled Abrogated Rad17 Regulation and Carcinogenesis in the Discussion. This is a serious over-interpretation of the date.

All they have done is shown that tumor samples have increased levels of Rad17, a previously reported finding. They have not shown any link between alterations in Rad17 stability and these changes in tumors. The data and the discussion of the data should be removed from the manuscript.

2nd Revision - authors' response 04 March 2010

Response to Editor

Our original referees 1 and 2 have now seen it again. In general, the referees are now positive about publication of your paper. However, both referees feel that there are a few issues that still need to be addressed (see below) before we can ultimately accept your manuscript. I would therefore like to ask you to deal with the issues raised. Given that both referees raise concerns regarding the tumor link put forward in figure 7, I would like to ask you to remove these data and the discussion referring to this issue.

-We agree with the suggestion from the editor and have removed the data concerning the tumor link in Figure 8A-C. The revised manuscript now focuses on the mechanism by which Rad17 is destroyed in response to UV radiation and further explores the relevance of its degradation in cellular recovery from genotoxic stress. We will leave the part concerning the possible impact of Rad17 proteolysis in carcinogenesis as a future study.

Due to the quality of Figure 3E, we are suggested to send a new manuscript that contains a suitably amended version of Figure 3E.

-The experiment design for Figure 3E was challenged by reviewer #1. We addressed the question in our previous rebuttal, but unfortunately, it did not satisfy the reviewer. In the new comments, the reviewer #1 again asked us to re-do this experiment including a demonstration of the abundance of Flag-ATR present on the IP-complex. As shown in the revised manuscript, we re-performed the experiment of Figure 3E exactly following suggestion from reviewer #1. We hope the quality of the new Figure 3E now comports with the editorial standard.

Response to Reviewer #1

In the revised version of the manuscript by Zhang et al, the authors address the more mechanistic details of proteolysis of Rad17 and found Cdh1/APC ubiquitin ligase as an interactor of Rad17, suggesting that Cdh1/APC might regulate Rad17 levels in response to UV. Interestingly, Cdh1 levels are regulated in response to UV and the downregulation of Cdh1 by shRNA affects Rad17 stabilization and degradation (figure 5H, which should be quantified). However, these experiments also raise additional questions. Given the fact that Cdh1 is strongly regulated throughout the cell cycle, one needs to determine whether the effect of Cdh1 downregulation on Rad17 levels is not merely a cell cycle effect. Alternatively, to strengthen the data that Cdh1 regulates Rad17 ubiquitination, the ubiquitination assay in figure 2D needs to be performed in the absence of Cdh1.

-Following the suggestion from the reviewer, we plotted the effect of Cdh1 depletion on UV-induced Rad17 degradation as shown in Figure 5H. In addition, we analyzed the effect of Cdh1 depletion on UV-induced Rad17 ubiquitylation as requested by the reviewer and indicated in new Figure 5I.

Although the manuscript is significantly improved by the addition of the new data, I am disappointed by the way the authors addressed the majority of the other points I addressed. I would therefore strongly recommend that the following issues are (re)addressed before this paper is published in EMBO Journal:

1) A major issue was whether Rad17 degradation is dependent on ATR-mediated phosphorylation, which is suggested by the experiments shown in supplemental figure 1. The authors argue that addressing this question is a difficult one, due to the lack of an appropriate assay, an in vitro

ubiquitylation assay. However, this issue can easily be addressed by determining the effect of UV light on the proteins of a Rad17-S635A/S645A phosphorylation mutant as compared to wild type Rad17, in an experiment as performed in Figure 4D. This mutant was actually published by one of the authors (XF Wang) in an elegant Nature paper (Bao et al, 2001), so should be readily available to the lab.

- We misunderstood the suggestion from the reviewer in regards to testing whether Rad17 degradation is dependent on ATR-mediated phosphorylation in our first revision. We appreciate the reviewer's guidance for this experimental design and have re-performed this experiment in the revised Supplemental Figure 1.

2) Figure 4B: Rad17 degradation mutants are made and their stability upon UV damage are tested. I asked to demonstrate the levels of each mutant before the induction of DNA damage, but the authors argue that the aim of the experiment is to determine the protein stability in response to UV. The aim of the experiment is very clear to me but I think the control of the expression levels of these mutants before damage induction is essential to be able to draw any conclusions about these constructs, as it is well known that some (deletion) constructs do not express well/differently due to folding problems. The experiment in Figure 4B should therefore contain an additional lane showing levels of the Rad17 deletion constructs at t=0.

-We agree with the reviewer's argument that the mutants could have differential expression. Following the reviewer's suggestion, we have reorganized the Figure 4 and included the expression of the set of Rad17 mutants at time zero.

3) Throughout the manuscript many co-immunoprecipitation experiments are shown in which the authors show interactions of (for example) Rad17 with other proteins. Loading controls of input are shown but for me it is essential also to see the amount of protein that is immunoprecipitated, and not only the interacting protein (Figure 3D: Rad9, Figure 3E: ATR, Figure 5A/B: Rad9, and Figure 6B: Rad9). The authors argue that these experiments are difficult to do due to a number of reasons: Rad9 runs at the height of the heavy chain and ATR is too big to see on the same gel. My suggestions for solving these problems: there are a number of good commercial anti-Rad9 antibodies of different species available. Perform the immunoprecipitation of Rad9 with a rabbit antibody, and the following western blot with a mouse of goat antibody. With respect to ATR: split the Flag-ATR IP sample into two, and run half on a gel to demonstrate Rad17 and the other half for immunoblotting for ATR or Flag

-We agree with the reviewer's argument that showing the loading control may not reflect the abundance of Rad9 and Flag ATR associated with the IP-complex (Figure 3D: Rad9, Figure 3E: ATR, Figure 5A/B: Rad9, and Figure 6B: Rad9). We re-performed experiment as shown in Figure 3D to show the amount of Rad9 on the IP-complex (this experimental design in Figure 3D is similar to the experiments in Figure 5A/B and Figure 6B, so we hope that the reviewer will agree with us that the status of Rad9 in Figure 3D could reflect that of Rad9 in Figure 5A/B and Figure 6B) as well as the abundance of Flag-ATR associating with the IP-complex. Please see revised Figure 3D & E.

4) The authors seem to have misunderstood my question about the graph of figure 3B. This graph now shows 2 smooth lines, which presumably fit the actual data points. I would like to see where in the lines the data points are situated, like has been done in figure 1C, 5F and 7E.

-We replotted Figure 3B following the reviewer's request. Please see the revised Figure 3B.

5) Finally, I am still uncertain whether the data describing the accumulation of Rad17 protein levels in tumour samples (Figure 7) is a valuable addition to this manuscript. To my suggestion to study the half-life of Rad17 in tumour cell lines the authors responded that this point is out of the scope of the current paper. I might agree with this but think that without such analysis, the performed data are too limited to draw a strong conclusion and would suit better in a separate manuscript discussing Rad17 levels in cancer in more detail.

-We agree with reviewer's argument, and we now only focus in the current manuscript on elucidating the mechanism by which Rad17 is destroyed in response to UV radiation and further explore its relevance in the cellular recovery from genotoxic stress. We removed the possible connection between Rad17 proteolysis and carcinogenesis in Figure 8A-C.

Response to Reviewer #2

1) In Point #4, reviewer 2 questioned why polyubiquitinated Rad17 did not accumulate in Fig. 2C. Although the authors responded, the response does not fit what is observed. Both of the provided rationales (smeared Rad17 due to ubiquitination and reduced immunoreactivity) would lead to lower levels of unmodified Rad17 in cells treated with MG132. That is not what is seen. Some explanation is required.

-In the previous comment #4, the reviewer anticipated to see an accumulation of UV-induced Rad17 ubiquitin conjugates in the presence of MG-132 by running pure Western blot. In theory, blockage of proteasomal function by MG-132 would lead to an accumulation of Rad17 ubiuqitin conjugates. We addressed that the possible reason why such accumulation of Rad17 ubiquitin conjugates was not visualized in Figure 2C was potentially due to technical difficulty in detecting smeared and modified Rad17 due to diffused ubiquitin signal and the sensitivity of antibody and ECL reagents. Another possibility could be that the antibody could not recognize the antigen efficiently if the antigen is modified (changed in its configuration). The reviewer further argues if our explanation is the case, we should observe lower abundance of the unmodified Rad17 but not its accumulation. At this point, we assume that the metabolic production of Rad17 (Rad17 transcription and protein translation) is not affected by UV radiation. We speculate that accumulated Rad17 ubiquitin conjugates due to blockage of proteasomal activity potentially causes a negative feedback regulation that may reduce the efficiency of Rad17 ubiquitylation and could result in a temporal accumulation of unmodified substrate.

2) In Point #5, reviewer 2 requested that the authors show additional controls for Fig. 2D. The reviewer indicated that it was essential that the experiment contain a control in which the cells are transfected with Myc-Ub but no HA-Rad17. The author's response was to add Supplemental Fig. 3, but this figure did not show what was requested, thus leaving open the possibility that polyubiquitinated species that was observed may be due to the non-specific interactions of other cellular proteins that accumulated when cells were treated with MG132.

-To satisfy the issue in regards to the standard of control for the Rad17 ubiquitylation assay raised in previous and current comments, we re-performed the Rad17 ubiquitylation assay as demonstrated in the revised Figure 3D.

3) There remains a serious concern regarding the attempts of the authors to link their findings to carcinogenesis. There are three (at least occurrences). First, they specifically mention a connection in the last sentence of the Summary. Second, the title of the relevant section in the Results is Impaired Rad17 Proteolysis Is Involved in Carcinogenesis. Third, there is a section entitled Abrogated Rad17 Regulation and Carcinogenesis in the Discussion. This is a serious over-interpretation of the date. All they have done is shown that tumor samples have increased levels of Rad17, a previously reported finding. They have not shown any link between alterations in Rad17 stability and these changes in tumors. The data and the discussion of the data should be removed from the manuscript.

- The same question was raised by the first reviewer. We addressed this question in the above point #5.

