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APOBEC3A can activate the DNA damage response and cause cell cycle arrest

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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 24 October 2010

Thank you for the submission of your research manuscript to our editorial office. We have now received the evaluation of three expert reviewers on your study.

As the reports are pasted below I would prefer not to repeat them here in detail, but to only summarize the main points raised by the referees. You will see that, while all referees agree on the potential interest of the findings, they also feel that in some instances additional work is needed.

The main concern raised by all three referees is that the study relies on the use of artificial overexpression of APOBEC3A. They state that evidence needs to be provided that the levels of overexpressed A3A correspond to those of the endogenous enzyme and that it should be tested whether activation of endogenous A3A by IFNalpha also induces DNA damage. Along these lines, the referees also feel that more discussion on the physiological conditions under which A3A would act on genomic DNA is needed. Referee 1 feels that additional proof that A3A directly causes DNA damage should be provided and that the experiments with the A3A mutant would need to be improved to exclude that the lack of DNA damage induction seen with this mutant is simply due to altered subcellular localization of the protein. Referee 2 suggests to compare the DNA damage induced by overexpression of A3A with that induced by AID and referee 3 recommends to use an alternative way to measure cell cycle effects of A3A overexpression.

Overall, given these evaluations, the reviewers constructive comments and the potential interest of the study, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the referees (as outlined above and in their reports) must be addressed,

especially with regard to the concerns on the levels of A3A expression and the effects of endogenous A3A on genomic DNA.

I look forward to seeing a revised form of your manuscript when it is ready.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The work described in the manuscript by Landry et al. explores the novel role of APOBEC3A (A3A) in DNA damage. This study shows that expression of A3A in U2OS cells results in a DNA damage response (DDR), such as the induction of phosphorylation of histone variant H2AX in a cytidine deaminase-dependent manner. The authors also show the phosphorylation of other downstream kinases in the DDR pathway such as Nbs1, Chk2 and RPA. Finally, the study also provides evidence that A3A expression results in DNA breakage and cell cycle arrest.

In general, the experiments in the manuscript are well done and controlled. However, the manuscript lacks an explanation of the pathways and molecules involved, as well as an interpretation and discussion of the results. Overall expansion of the introduction and results and discussion sections with some discussion of the hallmarks of the DDR pathway and interpretation of their results in this context would improve the manuscript.

Specific comments:

1. While the data support a model in which A3A directly causes DNA damage, no direct evidence that this occurs is provided. Either additional experiments should be performed to demonstrate that this occurs, or the authors should tone down their statements in the abstract and discussion.
2. Although the authors state that their transfected cells express the A3 proteins at endogenous levels (p. 4), this data is not shown. The authors should provide a data that this is indeed the case. Anti-A3A antibodies that recognize endogenous A3A are available (Thielen et al. 2010).
3. There is no discussion of the context in which A3A would function in vivo in the DDR. Since A3A could be deleterious to the host genomic DNA, the authors should discuss what would be the effects of basal expression and any physiological conditions that would result in its induction. A3A is not expressed in all tissues, so what would be responsible for the DDR in cells in which it is not expressed?
4. Fig. 1B - all the plasmids seemed to induce gH2AX to some extent and the A3-containing plasmids did so more than the pcDNA control. Is it commonly known that DNA transfection induces DDR?
5. Since the authors do not directly demonstrate that A3A induces DNA damage, a critical experiment is the use of the deamination-defective A3A mutant D106. However, nuclear localization of the C106 mutant appears to be decreased compared to wild type in all the figures (e.g. Figs. 2A, 3A, S2A). If this is the case, the lack of induction of DDR with this mutant may not be due to lack of deaminase activity. Can the authors use an A3A mutant that does not localize to the nucleus? Conversely, what happens when other A3 proteins such as A3G are targeted to the nucleus?

Minor comments:

1. Quantification is only provided for some of the experiments; most of the microscopic data shows only a single or few stained cells. Quantification of all the experiments should be performed and could be included in the supplement.
2. Page 5, sentence 2 - please cite reference.
3. In the results section on page 4, authors mentioned that "similar results were obtained in other cell

lines (data not shown)" - name the cell lines
4. Through-out the paper, expand the abbreviations when used for the first time.
5. In results section "A3A catalytic activity is required for activation of a DDR" please provide more details about why different downstream targets were analyzed and also the A3A mutants.
6. In the results section "A3A induces DNA breaks" - explain why the controls DNAse I and CAMPT were used.
7. Again the possibility of the DNA damage by A3A and its link to the cell cycle arrest need be discussed in more detail.

Referee #2 (Remarks to the Author):

In this manuscript, the authors provided evidence that enforced
expression of APOBEC3A in U2OS cells leads to the creation of
chromosomal ds DNA breaks as judged by Western blot analysis of H2AX phosphorylation, the formation of gamma-H2AX foci and TUNEL staining. The indication, given that the foci are not obtained with an A3A C106S mutant or in the presence of Ugi, is that the foci result from breaks generated following the action of UNG at sites of C-deamination.
These are interesting observations.

ï It would have been nice to have seen evidence that UNG activity is actually inhibited in the Ugi transfected cells (a minor point)
ï It would be good if the authors could compare the foci obtained with APOBEC3A to those obtained with AID (the only member of the AID/APOBEC family known to work physiologically on endogenous DNA).

Whilst the results are potentially exciting (suggesting a conceivable role for A3A in oncogenesis and genome evolution and the possible need for mechanisms to regulate A3As potentially mutagenic/oncogenic effects)), the major caution is of course that the results described here are all a consequence of contrived A3A over-expression achieved by use of transfection of A3A-encoding vectors.

In fact, although the work described here extends significantly on the findings reported by Stenglein et al in NSMB earlier this year (in that Stenglein et al only reported the deamination of transfected as opposed to endogenous DNA), Stenglein et al did observe mutation of the transfected DNA following the presumed induction of endogenous A3Ain monocytes.

I recommend that the authors therefore:

1 Test whether gamma-H2AX foci or an increased level of H2AX phosphorylation results from inducing expression of endogenous A3A using alpha-interferon (to which it is exquisitely sensitive). Possibly looking at P53-deficient cells. Even if negative, the results of such a study should be included so that the likely contribution of endogenous A3A to genomic instability can be assessed. [Even if rare, that would not exclude the possibility that rare deamination of endogenous DNA by endogenous A3A could very occasionally lead to cancer, but it would at least enable the reader to gauge the frequency range for endogenous mutation by endogenous A3A.]

2 If foci are not observed following IFN-alpha treatment, the controlled negative results should nevertheless be included. And the wording of the title/abstract/text modified so as to make the caution clear,and not mislead a casual reader. In other words, the title should change to 'APOBEC3A can activate the DNA...' and, in the Abstract, 'We show that enforced expression of APOBEC3A.....'.
And equivalent modifications throughout the manuscript. The findings would still be interesting.

3 Although not an essential experiment, it would be interesting to know if A3A transfection leads to an increased mutation frequency as judged by, for example, an hprt-inactivation assay

Referee #3 (Remarks to the Author):

In their manuscript "APOBEC3A activates the DNA damage response and causes cell cycle arrest" Landry et al demonstrate that the cytidine deaminase APOBEC3A is capable of generating DNA breaks, leading to activation of damage-responsive pathways. The experiments described are, in general, convincing and well controlled. Overall, the results support their main conclusion that APOBEC3A can generate cellular DNA damage. This is a very nice manuscript that will add significantly to our understanding of the functioning of DNA cytidine deaminases. Specific comments are:

1. The authors rely on a transfection system to express APOBEC3A in U2OS cells that do not show significant basal expression. These experiments are appropriately controlled and the results are convincing. However, a concern is that this is a somewhat artificial system that may not precisely reflect native APOBEC3A activity under normal conditions. The authors should clearly discuss this caveat, and qualify their conclusions. Without additional experiments they can strongly conclude that APOBEC3A can damage DNA but they cannot, from the data shown, conclude that APOBEC3A under normal conditions does damage cellular DNA. Their conclusions in this context would be significantly strengthened by additional experiments to address native APOBEC3A functions (e.g. RNAi to inhibit physiological A3A). These experiments would enhance the present study, but are probably not crucial for publication, with the appropriately qualified conclusions, at this time.
2. Experiments are described on p.5 and in the supplementary material which show that neither ATM nor DNA-PKcs are required for phosphorylation if histone H2AX after transfection with APOBEC3A. The authors conclude that A3A must activate a DNA damage response by multiple kinases. However, this conclusion is not supported by the data. From the data shown, it is possible that multiple kinases are involved (as the authors claim), or that there is an alternative DNA damage response pathway that culminates in H2AX phosphorylation via a single kinase. While this point is not central to their overall conclusions, they give a more balanced interpretation of the data.
3. Given that A3A expression appears to induce both DNA damage and cell cycle arrest, it is possible that the majority of the observed damage relates to the cell cycle effect, and thus indirectly to A3A action. The authors show propidium iodide staining data which appears to show a significant G1 arrect in A3A-expressing cells. However, PI staining gives relatively poor resolution of cell-cycle transitions, and so it is possible that the apparent G1 peak in the flow cytograms really represents an early S-phase arrest. If so, the damage seen may result from stalled DNA replication forks, rather than directly as a result of A3A activity. Additional means of measuring cell cycle effects are warranted, to confirm the author's interpretation.
4. Additional discussion regarding possible physiological functions of A3A, especially as they may relate to the reported DNA damaging activities, would be appropriate

1st Revision - authors' response 04 February 2011

Thank you for the reviews of our manuscript. We are grateful to the referees for their careful analysis of our work and we are pleased that all three referees were positive and found our work to be of considerable interest. We have addressed their concerns in a revised manuscript that includes substantial new data. We hope you will now find our work suitable for publication in EMBO Reports.

REFEREE #1
This referee stated that the experiments we presented in the manuscript were well done and controlled. This referee recommended expanding the text with some discussion of the hallmarks of the DDR pathway. The DDR is now briefly introduced at the beginning of the results section, and because of character limitations we also refer the readers to a recent review on DDR signaling. Specific comments from this referee are addressed below:

1. The referee notes that while the data presented support a model in which A3A directly causes DNA damage, no direct evidence that this occurs is provided. As requested by the referee, we have revised the abstract and discussion to include appropriate conclusions.

2. The referee recommends that we present data demonstrating that the amount of A3A expressed in transfected cells is comparable to that observed in IFN-stimulated PBMCs. An additional piece of data clarifying this point has now been added to Figure 1 (panel C).
3. The referee notes that the possible effect of endogenous A3A expression on genomic DNA integrity in the context of physiological events is not discussed. We thank the referee for drawing our attention to this important point. We have appended the text with additional discussion on the regulation of endogenous A3A expression and potential consequences for the genomic integrity of the cell (pages 10-11). The referee also asks what would be responsible for activating the DDR in cells not expressing A3A. While our results suggest that deregulated A3A expression leads to cellular DNA damage and activation of the DDR, we do not wish to conclude that A3A is required for activation of the DDR. We have amended the text to clarify this point.
4. The referee notes that in Figure 1B, γH2AX levels appear to be affected by DNA transfection alone (empty vector). To examine the effects of transfection on the DDR we have quantified γH2AX positive cells in each of the immunofluorescence experiments. We have also included a GFP-transfected control sample to account for possible transfection-induced H2AX phosphorylation. Results are presented in supplemental information (Fig S1B and S1D-E) and show that transfection alone did not result in significant H2AX phosphorylation.
5. The referee raised concerns about the cellular nuclear localization of the deaminase-defective C106S mutant when compared to wild-type A3A. To assess the possible impact of this mutation on the sub-cellular localization of A3A, we have compared the protein levels in nuclear and cytoplasmic fractions of transfected cells. These results demonstrate that comparable levels of A3A and C106S accumulate in the nuclear fraction. To confirm that A3A induces DNA damage signaling in a deaminase-dependent fashion, we made use of another deaminase defective mutant (E72Q). These results are now presented in Figure 1E-F and show that the E72Q mutant is also unable to induce H2AX phosphorylation in transfected cells. In addition, the referee asked whether targeting APOBEC3G to the nucleus would result in H2AX phosphorylation. To address this point, we generated an A3G-NLS fusion protein and evaluated its ability to induce γH2AX foci by immunofluorescence. Transfection of A3G-NLS in U2OS cells did not result in increased H2AX foci when compared to wild-type A3G. These results are now presented in Figure S1D and S1E. These observations are consistent with the fact that γH2AX is barely detected in cells expressing A3B or A3C, which can both localize in the nucleus. We therefore conclude that in our system, A3A induces the strongest DDR.

Minor comments:

1. As suggested by the referee, all immunofluorescence experiments have been quantified, and the data are presented in supplemental information.
2. Page 5, sentence 2, a reference has been added as requested.
3. Other cell lines in which similar results were obtained upon A3A transfection are now mentioned in the text.
4. All abbreviations have been expanded.
5. The referee requested further explanations on why different downstream targets were analyzed in cells transfected with A3A and the C106S mutant. We chose to look at the localization and activation of different effectors of the DNA damage response to include downstream targets for specific cellular kinases. For technical reasons, some antibodies were exclusively used for immunofluorescence or Western blot analysis. Unfortunately, because of character limitations, we were unable to include an extended description of our analysis of the DDR. Instead, we now refer to a recent review of the subject (page 4).
6. The reasons for using DNAseI and camptothecin as controls in the TUNEL assay have been clarified in the text (page 7).
7. The effect of A3A expression on cell cycle progression has been evaluated more carefully and the text has been amended to include additional discussion of the potential implications of these observations (page 9-10).

REFEREE #2

This referee stated that our study was interesting and extended significantly on the findings recently reported by Stenglein et al (Nat Struct Mol Biol 17(2): 222-229). He/she raised several points that required clarification. These points are addressed below:

1. The referee notes that no control was provided to confirm that UNG activity was inhibited in UGI-transfected cells. To address this point, we generated a new stable cell line expressing UGI, and analyzed A3A-transfected cells for γH2AX staining by immunofluorescence and Western blot. Results from the experiments performed with this cell line are now presented in Figure 3 and demonstrate that stable UGI expression prevents the induction of DNA damage signaling in cells transfected with A3A. In addition, we show that UNG activity is undetectable in this cell line (Fig S3E).
2. Since AID is the only member of the APOBEC/AID family that acts physiologically on genomic DNA, the referee suggested evaluating its ability to induce DNA damage signaling to allow comparison with A3A. We have thus performed additional experiments in U2OS cells and have compared the number of cells showing positive γH2AX staining for A3A, AID, A3G and A3G-NLS by immunofluorescence. We show that in our system, A3A expression induces the strongest DDR. These results are presented in Figure S1D-E.
3. This reviewer suggested that we test whether an increased level of H2AX phosphorylation results from inducing expression of endogenous A3A using INFα. Preliminary experiments suggest that treating PBMCs with INFα can result in increased A3A expression and H2AX phosphorylation. However, we have observed some variability with different donors and have not succeeded in knocking down A3A in these cells, so we cannot yet conclude that the observed H2AX phosphorylation occurs directly as a result of A3A expression. We are currently developing different systems that will allow further analysis of this issue and this will be a future focus of our research.
4. The referee suggests that mutation frequencies be investigated by an HPRT-inactivation assay. Although we agree that the question is interesting, this type of assay is complicated by the effect of A3A expression on cell cycle progression, as demonstrated in Figure 4. We are developing different systems that would allow us to address this question but feel that these are outside the scope of the current manuscript. A better understanding of the mechanisms required to regulate A3A expression and/or to prevent its detrimental effects on integrity of the host cell genome is clearly needed and is the subject of ongoing investigations in the lab.

REFEREE #3

This referee stated that the experiments we describe are generally convincing and well controlled, and that our manuscript “will add significantly to the understanding of the functioning of DNA cytidine deaminases”. Specific comments from this referee are addressed below:

1. This referee expressed concerns on the use of a transfection system to express APOBEC3A. We agree with the reviewer that the presented results do not allow us to conclude definitively that APOBEC3A causes DNA damage under normal conditions. However, we now show that the level of A3A required to induce a DDR in U2OS cells is comparable to the physiological levels (Fig 1C). In addition, we have generated new inducible cell lines expressing A3A and showed that induction results in H2AX phosphorylation (Fig 1G-H). Preliminary results suggest that stimulation of PMBCs with INFα could result in a significant increase in both A3A expression and H2AX phosphorylation. We have not been able to knockdown A3A successfully in primary cells, and therefore cannot yet conclude that endogenous A3A expression is the cause of the H2AX phosphorylation. We are currently looking at different systems that would allow us to address this question. The reviewer stated that with appropriately qualified conclusions these experiments are not crucial for publication. We agree with the reviewer that we should discuss this caveat and have appended the text accordingly (see page 10).
2. This referee questioned our interpretation that multiple kinases are activated in cells expressing A3A. The referee argues that the data presented do not demonstrate that ATM or DNA-PKs are required for the phosphorylation of H2AX in response to A3A. We agree that we cannot exclude that other cellular kinases could be activated by A3A and have corrected the text to draw the appropriate conclusion (page 6).

3. The reviewer notes that the resolution of cell cycle analysis using PI staining might not be sufficient to distinguish a G1 arrest from an early-S arrest. We agree with the reviewer and thank him/her for this suggestion. In order to evaluate carefully the effect of A3A expression on the cell cycle, we made use of our new inducible cell lines expressing A3A and the C106S mutant. This system facilitates cell cycle analysis by allowing us to look at the entire population without having to select for the subgroup of transfected cells. The data obtained using this system are now presented in Figure 4 and demonstrate that A3A induces an early S phase arrest. This observation was further confirmed by monitoring BrdU incorporation in cells expressing A3A (Fig 4C).
4. The referee suggests including additional discussion regarding A3A's potential physiological functions and how they may be associated with its ability to induce DNA damage. We agree with the reviewer that this is an important point and have amended the text accordingly (page 10-11).

We have added extensive new data to address all the concerns of the referees and we feel that our study has been improved through the review process. We appreciate the referees' thorough and constructive comments and thank you very much for your careful consideration of this manuscript. We hope that you will now find the manuscript appropriate for publication in EMBO Reports and we look forward to hearing from you in the near future.

2nd Editorial Decision 24 February 2011

The paper has been re-reviewed by three original referees with no further comments.

We are pleased to inform you that your manuscript has been accepted for publication and will be included in the next available issue of EMBO reports.

Sincerely,

Editor
EMBO Reports