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AIF promotes chromatinolysis and caspase-independent programmed necrosis by interacting with histone H2AX

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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 02 November 2009

Thank you for submitting your research manuscript (EMBOJ-2009-72935) to our editorial office. I apologize for the slight delay in getting back to you with a response, but after having carefully read it and discussed it with the other members of our editorial team, I decided to additionally consult with an expert editorial advisor of the Journal. The outcome of these consultations is, I am afraid to say, not a positive one, as we all agree that we cannot offer to publish your manuscript.

Your study aims at understanding how apoptosis-inducing factor (AIF) exerts its downstream effects, such as chromatinolysis, in programmed necrosis/caspase-independent programmed cell death. We agree that this is a very important question, as still very little is known on how AIF acts after having accumulated in the nucleus. Here, you identify a new nuclear interaction of AIF with the DNA repair-linked histone H2AX upon programmed necrosis-inducing alkylating DNA damage, and provide evidence for both phosphorylation of H2AX as well as for its requirement downstream of AIF activation and translocation to induced the necrotic PCD. While we appreciate that this is potentially very interesting, we however note that H2AX has already previously been implicated in programmed cell death, and more importantly, that the possible mechanisms underlying AIF action through H2AX remain currently very much unclear. Given the importance of and interest in the topic, I decided to nevertheless also consult an expert editorial advisor, to exclude that we might underestimate the potential significance of your demonstration of a functionally important AIF interaction partner. However, I am afraid that also this expert, who is well-acquainted with both the field and the standards of the journal, came to the same conclusion that without further insight into how this novel interaction might promote events such as chromatinolysis the study must still be

considered somewhat to preliminary for a broad general journal such as The EMBO Journal. I therefore have to return the manuscript to you with the message that we will not be able to consider it further for publication, at least not at the present stage of analysis.

Please note that we publish only a small percentage of the many manuscripts submitted to The EMBO Journal, and that the editors have been instructed to subject only those to external review that are likely to obtain enthusiastic responses both from our reviewers and readers. I am sorry to disappoint you on this occasion and hope that this negative decision does not prevent you from considering our journal for publication of other studies in the future.

Yours sincerely,
Editor
The EMBO Journal

Additional Correspondence 03 November 2009

Thank you for your comments on our manuscript (ref: EMBOJ-2009-72935). We are pleased to learn that you and the editorial board saw the value of our study, making comments such as ³your study aims at understanding how apoptosis-inducing factor (AIF) exerts its downstream effects, such as chromatinolysis², ³we agree that this is a very important question, as still very little is known on how AIF acts after having accumulated in the nucleus² or ³you identify a new nuclear interaction of AIF with the DNA repair-linked histone H2AX upon programmed necrosis-inducing alkylating DNA damage, and provide evidence for both phosphorylation of H2AX as well as for its requirement downstream of AIF activation and translocation to induced the necrotic PCD². The unique question you raise deals with "the possible mechanisms underlying AIF action through H2AX". The expert editorial advisor you contacted formulated a very similar remark: "without further insight into how this novel interaction might promote events such as chromatinolysis the study must still be considered somewhat preliminary".

To provide further insight into how this novel AIF/H2AX interaction promotes chromatinolysis in caspase-independent PCD, we have complementary data indicating that the cooperation between AIF and H2AX is required to improve DNA accessibility to caspase-independent endonucleases, such as cyclophilinA (and not Endonuclease G, other nuclease implicated in this type of cell death). These results have been obtained by the use of recombinant proteins,a flow cytometry assessment, a cell-free in vitro system analysis, and MEFs (mouse embryonic fibroblasts) in which we downregulated cyclophilin A or Endonuclease G. By using this multi-parametric approach, we demonstrate that AIF interacts with H2AX to generate an active DNA-degrading enzyme complex that provokes chromatinolysis and programmed necrosis/caspase-independent programmed cell death. Very importantly, we also show that the C-terminal Proline-rich binding domain of AIF is critical in generating such DNA-degrading complex.

We think that the addition of these new exciting results that have recently been generated in our laboratory will contribute to making our paper more solid and worthy of publication in EMBO Journal. I would like to stress that our manuscript also provides a complete characterization and a new molecular definition of the mechanisms regulating programmed necrosis/caspase-independent programmed cell death, demonstrating that this type of death proceeds via the nuclear interplay between AIF and histone H2AX, identifying the residues implicated in the AIF/H2AX association, and describing for the first time the effector domain of AIF, a critical point in the understanding of the AIF apoptogenic function that could pave the way for the development of future pharmacological tools.

With this in mind, we would appreciate further consideration of a new version of our manuscript including these new data, which we believe will clarify the doubt that both you and the external advisor have raised.

I am truly grateful for your time and consideration of my request. I look forward to hearing from you.

Additional Correspondence 03 November 2009

Thank you for your additional communication regarding your submission EMBOJ-2009-72935. Indeed, it appears that the new data you mention would address the main concerns the editors and editorial advisors had in this case. I would therefore be happy to look at an extended manuscript containing these additional mechanistic results as a new submission. To keep within the length limits, it may be worth considering to streamline the part of the study that describes biophysical analysis and computational modeling of the H2AX-AIF interaction, which seems somewhat less informative than the functional data (i.e. requirement of H2AX, requirement of the H2AX-AIF interaction, and the new data...). When submitting such a reworked manuscript, please clearly indicate your previous submission and my invitation to resubmit at the beginning of your cover letter, to ensure that the manuscript will be assigned to me with priority.

With best regards,
Editor
The EMBO Journal

1st Revision (Re-submission) 16 November 2009

Please find herein the manuscript entitled "AIF promotes chromatinolysis and caspaseindependent programmed necrosis by interacting with histone H2AX" you had the opportunity to read in a previous version in October 2009. The authors of this paper declare that they have no competing financial interests.
According to our previous correspondence and your invitation to resubmit, we have added a set of experiments that provide a further insight into how the AIF/H2AX interaction promotes chromatinolysis and caspase-independent programmed cell death (see Figure 8 and Supplementary Figure 6 in the new version of the manuscript). By using a multiparametric approach, we demonstrate that AIF interacts with H2AX to generate an active DNA-degrading enzyme complex implicating AIF, H2AX, and the endonuclease cyclophilin A. Very importantly, we also show that the C-terminal Proline-rich binding domain of AIF is critical in generating such chromatinolytic complex. Moreover, as you suggested in your last email, we have reorganized the part of the study that describes biophysical analysis and computational modeling of the H2AX/AIF interaction. Overall, the new version of our manuscript provides now a complete characterization and a new molecular definition of the mechanisms regulating AIF-mediated programmed necrosis/caspase-independent programmed cell death.
We hope you find this version of our manuscript suitable for peer reviewing.

Thank you for your concern regarding this paper.

2nd Editorial Decision 10 December 2009

Thank you again for submitting your amended manuscript for consideration by The EMBO Journal. We have now received the reports of three expert reviewers, whose comments directly to the authors are copied below. As you will see, all reviewers consider your findings on the AIF-H2AX link in programmed necrosis interesting and potentially important. Still, they also raise a number of substantive issues that would need to be addressed before publication in The EMBO Journal may be warranted. Should you be able to satisfactorily improve these points, we should be able to consider a revised manuscript for publication. I would therefore like to invite you to prepare such a revision in the spirit of the referees' criticisms and suggestions. In this respect, I feel it will be of particular

importance to address the role of H2AX Ser139 (along the lines proposed by both referees 1 & 2), and to provide some further biochemical validation of the proposed AIF-H2AX-CypA DNA degradation complex (as asked by referee 2). A further main concern, reflected in the comments of referees 2 and 3, is the currently incomplete differentiation between apoptotic and necrotic processes, which would need to be clarified. Please let me add that it is EMBO Journal policy to allow a single round of major revision only, and that it will therefore be important to diligently answer to all the various experimental and editorial points raised at this stage if you wish the manuscript ultimately to be accepted. When preparing your letter of response, please also bear in mind that this will form part of the Review Process File, and will therefore be available online to the community in the case of publication. 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):

The authors have demonstrated the involvement of H2AX in the process of programmed necrosis, and clearly have gained considerable insight into this type of programmed cell death. But the role of H2AX in the process is unclear to this reviewer.

Point 1. The authors establish that H2AX is phosphorylated during MNNG-induced damge, but it is not clear whether phosphorylation is essential.
Point 2. Figure 2 visualizes how the authors see the interaction of H2AX and AIF. AIF is shown interacting with a hydrophobic region of H2AX, away from its C-terminus and the phosphorylation site. Their H2AX model was from nucleosome core particle data, but is this hydrophobic region exposed in chromatin? It would be useful to show AIF interacting with H2AX in the nucleosome instead of just H2AX. It is also interesting that this same region is present in H2A and H2AZ (by conserved sequence), but these two histone species appear not to be involved.

At least it would have been useful to include the MEF H2AX-null line supplemented with a serine-alanine substituted H2AX as well as the wild-type H2AX. This would indicate whether both the hydrophobic region and the serine phosphorylation site are necessary for programmed necrosis.

This sentence is unclear. "Molecular modeling supported the notion that AIF interacts with H2AX through its C- terminal PBD. Does "Its" refer to AIF or H2AX. Presumably "It s" refers to AIF, since a later sentence says "This core is followed by a C-terminal tail, which is not involved in the interaction with AIF (residues 85 to 142)." This raises more questions since it is the H2AX C-terminal tail that differentiates it from H2A.

Referee #2 (Remarks to the Author):

The manuscript "AIF promotes chromatinolysis and caspase-independent programmed necrosis by interacting with histone H2AX" by Artus et al presents data showing that H2AX interacts with AIF during programmed necrosis triggered by high doses of MNNG, and that this interaction is required for the action of the latent endonuclease cyclophilin A to promote chromatinolysis. AIF interacts with H2AX via its C-terminal Proline rich binding domain. H2AX is phosphorylated on Ser139 during programmed necrosis and is required for AIF-mediated chromatinolysis, but dispensable for PARP-1, calpains, Bax and AIF translocation. The authors propose a model in which the synchronized presence of AIF, H2AX and Cyclophilin A is required to trigger the DNA

fragmentation occuring during programmed necrosis.

The topic and obtained results are of great interest, highlighting the importance of the AIF/H2AX complex in caspase-independent cell death. The laboratory has strong expertise in the study of programmed cell death pathways. In this study, they provide evidence for the implication of H2AX in AIF-dependent cell death. However, a few severe technical shortcomings need to be addressed to support the suggested conclusions. Furthermore, understanding how H2AX acts to stimulate chromatinolysis would greatly strengthen the message of this manuscript. In particular, it would be useful to provide further data showing whether the Ser139 phosphorylation of H2AX is a prerequesite for the effect of H2AX on chromatinolysis.

Major points:

1. The major issue of this manuscript is the absence of data showing whether phosphorylation of Ser139 is involved in programmed necrosis. Apparently, this phosphorylation is not required for AIF translocation, but it is not shown whether it as an impact on chromatinolysis. This can be tested by reconstituting the H2AX-/- cells with point mutated H2AX cDNA. The authors have the material and technology to do so. This point is particularly important in light of the results by Lu et al (2006) Mol Cell 23, 121-132 that showed that Ser139 phosphorylation of H2AX is required to stimulate CAD endonuclease activity during apoptosis.

2. The complex between AIF/H2AX and CypA could have been studied more intensively. The authors rely on the different binding domains within AIF for H2AX and CypA respectively to propose that the proteins can bind simultaneously. Some biochemical experiments should be done to see for example whether the interaction between AIF and CypA is altered in the absence of H2AX.

Minor points:

1. The rationale of the MNNG treatment used (the kinetics) in the different experiments should be more explicit. It will help the reader to know why MNNG treatment is for 9h when looking for the interactions, and only 15 min when looking for example at H2AX phosphorylation. The explanation comes only with figure 6B that shows the kinetics of AIF translocation. In addition, the time of MNNG treatment should be clearly indicated in each figure.

2. Figure 1D: the colocalization between AIF and H2AX is not convincing. A better image should be provided and the individual channels should be presented (as well as Dapi staining).

3. The modelisation of the interaction between AIF and H2AX is highly speculative. What are the evidences for this model? Molecular biology with point mutations is needed to support such models. In the absence of such data, the Figure 2 C and D should be moved to the supplementary data and the conclusions of this modelisation must be strongly toned down.

4. Figure 2D: why not showing the curves obtained with PBD sequence? This result should be illustrated instead of figuring as data not shown.

5. The rationale for using staurosporine is not clear. Why the authors have not used low doses of MNNG since this also induces "classical" apoptosis? In addition, Lu et al (2006) Mol Cell 23, 121-132 showed that H2AX is required for DNA ladder formation during UVA-triggered apoptosis (whereas dispensable for caspase-3 activation). Therefore, it is surprising that the % of Tunnel positive cells in STS treated H2AX-/- cells is similar to that of wt cells. The authors should comment that point.

6. The first paragraph of the discussion should be rewritten. The authors cannot say that H2AX is a downstream effector of AIF. Ser139 phosphorylation is an early event occurring before AIF translocation. And then, AIF and H2AX could act in concert to stimulate CypA.

Additional points:

- introduction : explain what is "PS".
- Page 4, last paragraph: replace "nucleasome" with "nucleosome"

- Figure 2A: the Koff(1) should not be placed above the part of the curve showing association, and should be moved above the Koff(2).
- some references are incomplete:
- Wang Y, Dawson VL, Dawson TM (2009a) Poly(ADP-ribose) signals to mitochondrial AIF: A key event in parthanatos. Exp Neurol

- Wang Y, Kim NS, Li X, Greer PA, Koehler RC, Dawson VL, Dawson TM (2009b) Calpain Activation Is Not Required for Aif Translocation in Parp-1-Dependent Cell Death (Parthanatos). J Neurochem

Referee #3 (Remarks to the Author):

The manuscript by Artus et al demonstrates that MNNG-induced necrosis involves the interaction between AIF and H2AX in the nucleus. The authors further show that the AIF-H2AX complex somehow activates necrosis through cyclophilin A (Cyp-A). The findings can be potentially interesting. However, a major issue is that since AIF, H2AX and Cyp-A have all been implicated to regulate apoptosis, it is unclear whether these activation events associated with these molecules are uniquely required for programmed necrosis.

1. The authors set out to try to address the question of how AIF mediates necrotic cell death in response to MNNG. Since AIF, H2AX (Oncogene (2008) 27: 5662) and Cyp-A (Oncogene. 2004 Feb 26;23(8):1514-21; J Exp Med. 2007 Aug 6;204(8):1741-8) have all been implicated in apoptosis, the authors did not make a compelling case how this AIF-H2AX-Cyp-A molecular machinery is specifically turned on to induce necrosis. This is particularly a concern given that many of the results the authors show appears to be events activated in apoptosis as well (e.g. TUNEL staining, chromatin condensation, S139 phosphorylation on H2AX, etc.). The results will be more compelling if the authors can demonstrate that these events occur specifically in cells undergoing necrosis. For example, since MNNG can cause apoptotic or necrosis depending on the dose, do the authors know if the interaction among AIF, H2AX and Cyp-A only occurs with high doses of MNNG that trigger necrosis? What about using other agents that also induce DNA damages but are known apoptosis inducers (e.g. etoposide)?

2. The authors repeatedly refer to H2AX-/- cells being resistant to MNNG-induced necrosis. However, as the authors show in supplemental fig. 5A, the H2AX-/- cells eventually underwent death, albeit with a slower kinetics. In fact, these cells appears to be more "necrotic" than WT cells, which seems to argue against an obligate requirement for H2AX in the necrotic pathway that the authors were examining. The authors should at the very least try to discuss these issues.

3. The authors raised the interesting possibility of targeting AIF in cancer therapies at the end of the discussion. Can the authors demonstrate that the PBD peptide alone is sufficient to induce chromatinolysis in isolated nuclei?

Other comments:
1. Full length AIF should be shown in addition to tAIF in all figures.
2. The authors should quantify the results for PAR in Fig. 5B.
3. How did the authors purify AIF from the mitochondria and the inter-mitochondrial space (IMS) in Fig. 6B? Also, the authors should show by Western blots that they cytoplasmic extracts are devoid of nuclear proteins (e.g. lamin) and vice versa.
4. Whenever quantification was done with imaging assays, please provide information on the total number of cells examined (Figs. 3D, 4D, 5D, 6D, 7E and 8B-C).

2nd Revision - authors' response 09 February 2010

We thank the reviewers for the thoughtful insights that have resulted in a greatly improved

manuscript. Their constructive criticism has been fully taken into account in the revision of the manuscript (see our point-bypoint reply below). We have performed a substantial number of additional experiments and made changes in response to all the concerns.
Briefly:
- We now demonstrate the key role of H2AX Ser139 phosphorylation in caspase-independent programmed necrosis (new Figure 4E);
- We have studied the H2AX/AIF/CypA DNA degrading complex more in detail (new Supplementary Figures 8 and 9);
- We better explain the molecular modeling approach represented in Figures 2C and 2D;
- We long-establish, in the new Results and Discussion sections, the specificities of AIF and the programmed necrotic process described in our manuscript;
- Finally, we define a new AIF-derived peptide that, containing both the H2AX and CypA binding residues, is sufficient to provoke DNA degradation in purified nuclei (new Supplementary Figure 10).

REVIEWER #1
This reviewer said: "The authors have demonstrated the involvement of H2AX in the process of programmed necrosis, and clearly have gained considerable insight into this type of programmed cell death". Then, raised two major concerns:

1.- It would have been useful to include the MEF H2AX-null line supplemented with a serine-alanine substituted H2AX as well as the wild-type H2AX.

The referee suggested that we verify whether H2AX phosphorylation on Ser139 is essential in MNNGmediated programmed necrosis, and we have followed this suggestion. We mutated H2AXSer139 to Ala (S139A). Then, we transfected wild-type H2AX and H2AXS139A into H2AX -/- cells. As illustrated in the revised version of our manuscript, contrary to the transfection of wild-type H2AX, the mutated form H2AXS139A did not restore the MNNG ability to induce programmed necrosis in H2AX -/- cells. This demonstrated that H2AX phosphorylation on Ser139 is essential in this type of caspase-independent cell death, exactly as in UVA or etoposide-induced caspase-dependent cell death (Lu et al., 2006, Mol Cell vol. 23, pp. 121-132). The role of H2AX in programmed necrosis seems thus critical in the comprehension of the molecular mechanisms that control H2AX/AIF interaction, chromatinolysis and cell death after MNNGtreatment
(see also point 2 below).
These additional data on H2AX phosphorylation are presented in the new Fig. 4E and in the Results section (page 8, paragraph 1, lines 6-10). A new paragraph, highlighting the relevance of the generation of H2AX in MNNG-induced programmed cell death (PCD), is now included in the Discussion section (page 12, paragraph 3).

2.- Differences between H2AX and H2A/H2AZ. AIF/H2AX nucleosomal interaction.

i) The referee pointed out that H2A, H2AZ, and H2AX have a conserved sequence and that the hydrophobic region of H2AX interacting with AIF is preserved in all these variants. However, only H2AX seems implicated in the interaction with AIF. Indeed, the difference between H2AX and H2A/H2AZ is made by the specific characteristics of H2AX. First, its C-terminal tail makes H2AX unique (Redon et al. Curr Opin Genet Dev 2002, 12, 162-169). This tail includes the phosphorylatable Serine 139. Second, Serine 139 phosphorylation alters H2AX conformation into the nucleosome and the conformation of the nucleosome itself. Finally, H2AX presents a specific redistribution into the cellular volume. This redistribution helps in organizing specialized protein complexes (Bewersdorf et al. PNAS 2006, 103, 18137-18142). Together, these changes in H2AX/nucleosome elicit a modification in the structure and functionality of the chromatin fiber, making it accessible to diffusible factors (Paull et al. Curr Biol 2000, 10, 886-895; Thiriet & Hayes, Mol Cell 2005, 18, 617-622; Fernandez-Capetillo et al. Cell Cycle 2003, 2, 426-427; Ayoub et al. Cell Cycle 2009, 8, 1-7; Fernandez-Capetillo et al. DNA repair 2004, 3, 959-967; Sluss & Davies, Mol Cell 2006, 23, 152-153). Thus, even if H2A, H2AZ, and H2AX have a conserved sequence, only the posttranslational modification ensued by H2AX favors the exposure of its

hydrophobic region and the interaction/access of soluble proteins to chromatin or H2AX itself. This substantiates our new results on H2AX (see point 1 above) and the data included in old Figures 1 and 2 and the new Supplementary Figure 8, all of which illustrate the physical interaction between AIF and H2AX. Together these results support our molecular modeling approach (Figures 2C, 2D, and Supplementary Figure 9) (see also answer to Reviewer #2, minor comment iii) and confirm that both the H2AX hydrophobic region (by interacting with AIF) and the serine phosphorylation site (by favoring protein accessibility and changing chromatin conformation) are essential in programmed necrosis.
ii) Concerning the nuclear localization of the AIF/H2AX link, it seems interesting to underline that, following the generation of double strands breaks (DSB) in DNA, H2AX Ser139 is phosphorylated in nucleosomes near the DNA break point. Importantly, phosphorylated H2AX (H2AX) does not redistribute outside this nuclear structure (Rogakou et al. J Cell Biol 1999, 146, 905-16; Thiriet & Hayes, Mol Cell 2005, 18, 617-622; Fernandez-Capetillo et al. Cell Cycle 2003, 2, 426-427; Fernandez-Capetillo et al. DNA repair 2004, 3, 959-967). In our manuscript, we demonstrate by a triple assessment (flow cytometry, immunofluorescence, and immunoblotting), that high doses after MNNG generate H2AX (Figures 3A, 3B, and 3C). This phosphorylation is observed as soon as 5 min after MNNG treatment. Strikingly, H2AX remains phosphorylated even 9 h post MNNG treatment (new Figure 3C). Additionally, we demonstrate that AIF translocates from the mitochondria to the nucleus 6/9 h after the treatment of cells with MNNG (Moubarak et al. Mol Cell Biol 2007, 27, 4844-4862 and Figures 6B and 6C). Thus, in arriving at the nucleus, AIF finds H2AX in the nucleosome.
According to our new and previous results (Figures 1, 2, 3C, 4E, 6B, 6C and Supplementary Figure 8), we have added two new paragraphs to the Introduction and Discussion sections in which we state the particular characteristics of H2AX and the relevance of H2AX in programmed necrosis (page 4, paragraph 3 and page 12, paragraph 3).

Minor comments:
i) This sentence is unclear "Molecular modeling supported …".
Following the reviewer's suggestion, we have amended the sentence (page 6, paragraph 3, lines 1-3).

REVIEWER #2
This referee stated: "The topic and obtained results are of great interest, highlighting the importance of the AIF/H2AX complex in caspase-independent cell death. The laboratory has strong expertise in the study of programmed cell death pathways. In this study, they provide evidence for the implication of H2AX in AIFdependent cell death". Then, commented:

1.- The major issue of this manuscript is the absence of data showing whether phosphorylation of Ser139 is involved in programmed necrosis.

As requested by this reviewer and Reviewer #1, in the new version of our manuscript we have included data showing the relevance of H2AX Ser139 phosphorylation in programmed necrosis (see also response to Reviewer #1, specific point 1).

We have performed new experiments in which H2AX knockout MEFs have been transfected with a serine-alanine substituted H2AX (S139A) as well as the wild-type H2AX. As indicated above, contrary to the wild-type H2AX, mutated H2AXS139A does not restore the MNNG ability to induce programmed necrosis in H2AX -/- cells. Therefore, similarly to what has been previously described for caspase-dependent programmed cell death (Lu et al., 2006, Mol Cell vol. 23, pp. 121-132), the generation of H2AX is critical in caspase-independent programmed necrosis. As described in the Discussion section, it seems that both the particular redistribution of phosphorylated H2AX (H2AX) within the nuclear volume and the DNA restructuration induced by H2AX are essential for the AIF/CypA action that promotes caspase-independent DNA degradation. Importantly enough, our new data demonstrate that chromatinolysis promoted by H2AX is a common feature in caspase-dependent and caspase-independent PCD. The difference in the nature of the DNA degradation representing these two types of death (oligonucleosomal for caspase-dependent PCD and

large scale for caspase-independent PCD) seems relied to the DNases implicated: CAD or cyclophilin A (CypA), respectively.
As indicated in the response to Reviewer #1, our new data on S139-mutated H2AX are presented in Figure 4E. Results and Discussion sections have been modified accordingly.

2.- The complex between AIF/H2AX and CypA could have been studied more intensively. The authors rely on the different binding domains within AIF for H2AX and CypA respectively to propose that the proteins can bind simultaneously. Some biochemical experiments should be done to see for example whether the interaction between AIF and CypA is altered in the absence of H2AX.

Following the referee's suggestion, we have performed a double immunoprecipitation assay to comparatively analyze the binding of the endogenous proteins AIF, H2AX and CypA in programmed necrosis. Our working hypothesis is that AIF could simultaneously interact with H2AX and CypA in the nucleus of MNNG-treated cells. In this way, extracts from highly purified nuclei obtained from WT MEFs treated or not with MNNG (9 h) were subjected to H2AX and CypA immunoprecipitation. As expected, AIF did not co-immunoprecipitate with H2AX or CypA in control MEFs. However, treatment with MNNG led to the association of AIF with H2AX and CypA (Supplementary Figure 8). These original results for the first time demonstrated that AIF could interact with H2AX and CypA simultaneously in programmed necrotic conditions.
In a second set of experiments, we evaluated whether the interaction between AIF and H2AX is altered in the absence of CypA and whether the association between AIF and CypA is perturbed in the absence of H2AX. To do that, we purified nuclei from CypA downregulated cells and from H2AX -/- MEFs treated or not with MNNG (9 h), and performed a similar immunoprecipitation assay to that described above. As shown in Supplementary Figure 8, the AIF/H2AX link remained undisturbed in the absence of CypA (immunoprecipitations performed in extracts from nuclei purified from CypA downregulated cells).
On the contrary, AIF needs H2AX to associate with CypA in the nucleus (AIF does not coimmunoprecipitate with CypA in H2AX -/- cells treated with MNNG). These results indicate that the presence of H2AX in the nucleus is crucial for the interaction of AIF with CypA. This underlines the potential role of the AIF/H2AX link in programmed necrosis. With this in mind, we propose that the AIF/H2AX interaction favors the further association of AIF with CypA. This is illustrated in a theoretical model represented in Supplementary Figure 9. In this Figure, we propose that the interaction between AIF and H2AX stabilizes the AIF/CypA link.
We have included these new data in Supplementary Figures 8 and 9. In the Discussion section of our revised manuscript, we now state that "AIF could simultaneously associate with H2AX and CypA" and that "AIF interacts with H2AX even in the absence of CypA. In contrast, AIF needs H2AX in the nucleus to associate with CypA" (page 13, paragraph 2, lines 15-21). The Supplementary Materials and Methods section has been modified accordingly.

Minor comments:
i) The rationale of the MNNG treatment used (the kinetics) in the different experiments should be more explicit. It will help the reader to know why MNNG treatment is for 9h when looking for the interactions, and only 15 min when looking for example at H2AX phosphorylation.

In line with the reviewer's point, a sentence now explains the rationale of the MNNG-treatment used in our manuscript (page 5, paragraph 2, lines 1-3). Indeed, this rationale was obtained from our recent published work (Moubarak et al. Mol Cell Biol 2007, 27, 4844-4862), which is cited in the original version of our manuscript (page 5, paragraph 1, line 7) and represented in Figure 1A. In this previous paper, we demonstrate that the proapoptotic form of AIF redistributes from the mitochondria to the nucleus 6/9 h post- MNNG treatment to induce chromatinolysis and cell death. Thus, we have used this timeframe to identify nuclear components downstream of AIF in programmed necrosis. Note that the timeframe of the mitochondrio/nuclear redistribution of AIF has been corroborated in H2AX-wt and H2AX -/- MEFs (Figure 6B and 6C). Concerning H2AX, in the new version of our manuscript we have included a complete kinetic analysis (from 5 minutes to 9 h post MNNG treatment). This assessment demonstrated that H2AX phosphorylation is a rapid and time-dependent process observed as soon as 5 min after MNNG treatment.
Importantly, H2AX remains phosphorylated even at 9 h post MNNG treatment (new Figure 3C). Thus, when AIF arrives at the nucleus, it finds H2AX (see also response to Reviewer #1, specific

point 2).

ii) Figure 1D: the colocalization between AIF and H2AX is not convincing. A better image should be provided and the individual channels should be presented (as well as Dapi staining).

The old Figure 1D has been improved according to the reviewer's proposal: AIF and H2AX are provided in individual channels and a nuclear staining (Hoechst 33342) has now been included.

iii) The modelisation of the interaction between AIF and H2AX is highly speculative. What are the evidences for this model?

We agree with the referee that a molecular model is a theoretical representation. However, this model has a special importance in our manuscript. In fact, Figures 2C and 2D illustrate how the structural and dynamic properties of AIF and H2AX could determine their interaction. In this way, in our molecular modeling approach, the affinity and kinetic results obtained by surface plasmon resonance (Figures 2A and 2B) have been taken into consideration: (a) the N-terminal domain of AIF does not interact with H2AX; (b) AIFsh, the C-terminal domain of AIF, and AIF's C-terminal PBD present high affinity for H2AX. Using these premises, our 3D modeling not only represents a theoretical AIF/H2AX link, but Figures 2C and 2D also corroborate that the link between AIF's C-terminal PBD and H2AX is possible. This is further substantiated by mutagenesis (as requested by this referee): substitution of the five prolines of the PBD motif by alanines eliminated the interaction of AIF's C-terminal PBD with H2AX (Figure 2B). As a result of the biophysical and molecular biology results, we prefer to maintain Figures 2C and 2D in the manuscript rather than move them to the Supplementary information section. We have modified the Results section of our manuscript to include a sentence indicating the evidences used in the in silico prediction of the quaternary structure. Moreover, as requested by this referee, we have toned down the conclusions obtained with our molecular modeling approach and now we specify that the model "illustrates" the AIF/H2AX association (page 6, paragraph 3, lines 1-3).

iv) Why not showing the curves obtained with PBD sequence? This result should be illustrated instead of figuring as data not shown.

Following the referee's proposal, we have amended this Figure, which now includes the curves obtained with the PBD and PEST sequences.

v) The rationale for using staurosporine is not clear.
In Figure 4A we demonstrated that, compared to WT cells, the H2AX knockout MEFs present lower responsiveness to MNNG treatment. These data raised a complementary question: Are the H2AX -/- MEFs sensitive to other cell death programs? To answer this question we used a positive control, the classical caspase-dependent apoptotic inducer staurosporine (STS). Note that, as reported in a recent manuscript (MuÒoz-Pinedo et al. Proc Natl Acad Sci 2006, 103, 1573-1578), STS induces caspase-dependent apoptosis, and it does so without implication of AIF. STS and MNNG therefore represent two different forms of programmed cell death.
Thus, we have simultaneously treated WT and H2AX -/- MEFs with high doses of MNNG (to induce AIF-mediated caspase-independent programmed necrosis) and with STS (to induce caspasedependent cell death). As depicted in Figure 4A, contrary to the response observed with MNNG treatment, WT and H2AX -/- MEFs treated with STS displayed similar cell death levels. H2AX knockout cells remain therefore sensitive to caspase-dependent apoptosis inducers, such as STS, even if they present lower responsiveness to MNNG-mediated programmed necrosis. This confirms previously published results indicating that H2AX -/- MEFs are sensitive to STS treatment (Mukherjee et al. DNA repair 2006, 5, 575- 590). Importantly, together with our previous results (Moubarak et al. Mol Cell Biol 2007, 27, 4844-4862), these new data corroborate the existence of an AIF necrotic pathway that could be regulated independently of the classical caspase-dependent cell death pathway represented by STS.
We have modified the Results section of our manuscript to include a sentence indicating that "H2AX -/- MEFs remained sensitive to STS-mediated death (Mukherjee et al. 2006)" (page 7,

paragraph 3, lines 10- 13). Moreover, we have included in the Discussion section a sentence stating that: "nitrosoureas engage a highly regulated necrotic pathway that could be modulated independently of classical apoptotic pathways" (page 14, paragraph 3, lines 1-2).

vi) The first paragraph of the discussion should be rewritten.
In agreement with this referee, we have revised the first paragraph of the Discussion section.
Additional points: The text, Figures, and References of the present version of our manuscript have been modified according to the Reviewer's proposal.

REVIEWER #3
This reviewer said: "The manuscript by Artus et al demonstrates that MNNG-induced necrosis involves the interaction between AIF and H2AX in the nucleus. The authors further show that the AIF-H2AX complex somehow activates necrosis through cyclophilin A (Cyp-A). The findings can be potentially interesting".
Then, pointed out the following major issues:
1.- The authors set out to try to address the question of how AIF mediates necrotic cell death in response to MNNG. Since AIF, H2AX (Oncogene (2008) 27: 5662) and Cyp-A (Oncogene. 2004 Feb 26;23(8):1514-21; J Exp Med. 2007 Aug 6;204(8):1741-8) have all been implicated in apoptosis, the authors did not make a compelling case of how this AIF-H2AX-Cyp-A molecular machinery is specifically turned on to induce necrosis. For example, since MNNG can cause apoptotic or necrosis depending on the dose, do the authors know if the interaction among AIF, H2AX and Cyp-A only occurs with high doses of MNNG that trigger necrosis? What about using other agents that also induce DNA damages but are known apoptosis inducers (e.g. etoposide)?

We agree with this reviewer that proteins implicated in caspase-independent programmed necrosis also participate in caspase-dependent apoptosis. In fact, the specificity of the caspase-independent necrotic program described in the present manuscript is determined by:
(a) The disproportionate activation of PARP-1 induced by high doses of MNNG.
Activation of PARP-1 results in poly(ADP-ribosyl)ation of key DNA-repair proteins at the expense of NAD+ that is cleaved into ADP-ribose and nicotinamide (Heince et al. Trends Mol Med 2005, 11, 456-463; Shall et al. Mutat Res 2000, 460, 1-15). When DNA-damage is limited (e.g., MNNG treatment at 0.5-10 M), this physiological machinery, which normally plays homeostatic protective and regulatory roles, could repair the injury. If DNA breaks are repaired, the damaged cells survive and the cellular NAD+ levels are restored by recycling nicotinamide with two ATP molecules. If DNA repair is not completely achieved, cells undergo apoptosis by a caspase-dependent mechanism. When DNA-damage is extensive (e.g. MNNG treatment at 100-500 M), cells cannot repair the injury. In this case, disproportionate activation of PARP- 1 depletes the cellular pools of NAD+ and ATP, driving the cell to a necrotic type of PCD. Therefore, MNNG could induce apoptosis or programmed necrosis depending on the level of the DNA-damage generated. This is illustrated in Figure 1A and was extensively described in Moubarak et al. Mol Cell Biol 2007, 27, 4844-4862, Zhong et al. Genes Dev 2004, 18, 1272-1282, and Yu et al. Science 2002, 297, 259- 263 (references cited in the manuscript).
(b) The release of AIF from mitochondria to cytosol and nucleus. The presence of AIF in the nucleus is essential in the generation of a DNA-degrading complex with the nuclear proteins H2AX and CypA. In this sense, it is important to remark that the mitochondrio/nuclear redistribution of AIF is restricted to caspase-independent cell death paradigms, such as programmed necrosis.
- The mitochondrial processing and release of AIF is mediated by calpains or cathepsins, not by caspases (Otera et al, EMBO J 2005, 24, 1375-1386; Polster et al, J Biol Chem 2005, 280, 6447-6454; Yuste et al, Cell Death Differ 2005, 12, 1445-1448; Liu et al. Int J Cancer 2009, 125, 2757-2766; Cao et al. J Neurosci. 2007, 27, 9278-9293). Indeed, the caspase-independent mitochondrial release, nuclear translocation, and DNA fragmentation associated with AIF have all been extensively demonstrated in several systems and cell types (~900 references in Medline to date) (e.g., Constantinou et al. Curr Cancer Drug Targets 2009, 9, 717-728; Joza et al. Ann N Y Acad Sci 2009, 1171: 2-11; Lorenzo & Susin, FEBS Lett 2004, 557, 14-20; Cande et al. J Cell Sci 2002, 115, 4727-4734; Modjtahedi et al. 2006, Trends Cell Biol 16, 264-272; Hong et al. Trends Pharmacol Sci 2004, 25, 259-264; van Gurp et al. Biochem Biophys Res Commun 2003, 304, 487-497; Hansen & Nagley, Sci STKE 2003, 193, PE31; Lorenzo & Susin, Drug Resist Updat 2007, 10, 235-255; Lorenzo HK et al. Cell Death Differ 1999, 6, 516-24). This broad bibliographic support corroborates

that AIF is exclusively a caspase-independent cell death effector. - In apoptotic caspase-dependent PCD, the absence of relevant mitochondrial AIF release disclose the participation of this protein in the execution of cell death, making the formation of the H2AX/AIF/CypA complex described in the present work unfeasible. As stated in a recent manuscript: "The timing and extent of AIF release makes it unlikely that it is involved in the induction of apoptosis, either upstream or downstream of mitochondrial outer membrane permeabilization" (MuÒoz-Pinedo et al. Proc Natl Acad Sci 2006, 103, 1573-1578). In this sense, the role of AIF in MNNG or etoposide-mediated apoptosis does not seem relevant. In fact, it has been demonstrated that these two PCD programs are regulated by caspases and the Caspase-Activated DNase -CAD- (e.g., Lu et al., 2006, Mol Cell vol. 23, pp. 121-132; McIlroy et al. Oncogene 1999, 18, 4401-4408; Meador et al. Oncogene 2008, 27, 5662-5671).
- Contrary to etoposide or MNNG-mediated apoptosis, programmed necrosis is an AIF-dependent and caspase-independent PCD program. AIF downregulation or neutralizing anti-AIF antibodies inhibit MNNG-induced programmed necrosis (Moubarak et al. Mol Cell Biol 2007, 27, 4844-4862, and Yu et al. Science 2002, 297, 259-263). Specific or broad caspase-inhibitors do not block programmed necrosis (Zhong et al. Genes Dev 2004, 18, 1272-1282; Zhong & Thompson, Genes Dev 2006, 20, 1-15; Yu et al. Science 2002, 297, 259-263; Boujrad et al. Cell Cycle 2007, 6, 2612-2619; Wsierska-Gadek et al. Ann N Y Acad Sci 2003, 1010, 278-282; Moubarak et al. Mol Cell Biol 2007, 27, 4844-4862, and Supplementary Figure 1).

Moreover, the presence of AIF in the nucleus is essential in programmed necrosis (Moubarak et al. Mol Cell Biol 2007, 27, 4844-4862; Yu et al. Science 2002, 297, 259-263; Ethier et al. Apoptosis, 2007, 12, 2037-2049). This is confirmed in the old version of our manuscript (Figures 1, 6, 7, 8, and Supplementary Figure 6) and corroborated by our new results, thereby indicating that AIF provides an essential bridge between H2AX and CypA and that the AIF/H2AX link promotes chromatinolysis by favoring the interaction of AIF with CypA (Supplementary Figures 8, 9, and 10) (see also response to Reviewer #2, specific point 2 and this reviewer, specific point 3). Overall, our data establish that the formation of the H2AX/AIF/CypA complex is critical in programmed necrosis. Contrary to apoptotic etoposide or MNNG-mediated PCD, the caspase-independent AIF release associated to programmed necrosis favors the formation of such DNA-degrading complex. According to this reviewer, the new version of our manuscript reveals that: "implicating similar effectors (e.g., H2AX, PARP-1, or Bax), the apoptotic and necrotic pathways could represent alternate outcomes of a similar PCD program. In fact, the unlike activation of PARP-1 and the implication of caspases or AIF seem the main differences between these two PCD modalities". We have clarified this issue in a new paragraph included in the Discussion section (page 12, paragraph 2) and included a sentence in the Results section: "when the DNA damage is extensive, the cell undertakes a caspase-independent PCD program called programmed necrosis" (page 5, paragraph 1, lines 2-4). Moreover, the specificities of the DNA-degrading complex that controls chromatinolysis and programmed necrosis are now described on pages 12 and 13 and the potential role of each component of the H2AX/AIF/CypA complex is enlightened on page 14, paragraph 1, lines 3-6.

2.- The authors repeatedly refer to H2AX-/- cells being resistant to MNNG-induced necrosis. However, as the authors show in supplemental fig. 5A, the H2AX-/- cells eventually underwent death, albeit with slower kinetics.

The concern regarding cell death in H2AX-/- cells is well justified and we have eliminated the term "resistant". However, note that compared to H2AX-wt (WT) cells, the type of death induced by high doses of MNNG in H2AX-/- MEFs is not exactly the same:
- On the one hand, H2AX knockout cells presented similar PARP-1, calpains, Bax, and AIF activation kinetics to WT cells. In both cell types, after MNNG treatment, the mitochondrion was altered and the NAD+ and ATP pools were quickly consumed (Figures 5, 6, and Supplementary Figures 3 and 4).
- On the other hand, H2AX -/- cells only presented a significant positive PS exposure/loss of viability labeling 24 h post MNNG incubation. In contrast, WT MEFs reached a similar % of PS exposure/loss of viability 9 h after MNNG treatment (Figure 4B). More importantly, contrary to WT cells, H2AX knockout MEFs never presented the hallmarks characterizing AIF-mediated chromatinolysis, and 24 h post MNNG
treatment they exploded, as in uncontrolled necrosis (Supplementary Figures 5B and 5C). This key difference underlines the role of DNA degradation, promoted by the AIF/H2AX link, in

programmed necrosis.
We have added a sentence in the manuscript stating that: "DNA chromatinolysis is a major step in programmed necrosis. Without the DNA degradation promoted by this link, the MNNG necrotic program remains unachieved and the cells explode" (page 12, paragraph 2, lines 5-7).

3.- The authors raised the interesting possibility of targeting AIF in cancer therapies at the end of the discussion. Can the authors demonstrate that the PBD peptide alone is sufficient to induce chromatinolysis in isolated nuclei?

In line with the referee's suggestion and with our new results indicating that AIF provides an essential bridge between H2AX and CypA (Supplementary Figures 8 and 9; see also response to Reviewer #2, specific point
2), we have synthesized and tested in our cell free system three different AIF-derived peptides containing either the PBD or the CypA binding domains, or the two domains (AIF PBD, AIF CypA, and AIF PBD/CypA, respectively). As depicted in the new Supplementary Figure 10, only the AIF-derived peptide containing the PBD and the CypA binding sites (AIF PBD/CypA) provokes DNA degradation in purified nuclei. In contrast, AIF-derived peptides containing either the PBD or the CypA binding sites are unable to induce chromatinolysis. This corroborates that AIF needs its H2AX and CypA binding sites to promote DNA degradation. Importantly enough, our new results explain the absence of chromatinolytic activity in some of the previously described AIF forms: AIFsh2 (Delettre at al. J Biol Chem 2006, 281, 18507-18518), which lacks the H2AX and CypA binding domains; AIF 263-399 (Cande et al. Oncogene 2004, 23, 1514-1521), which lacks the CypA binding domain, or tAIF Pro-rich and AIFsh Pro-rich (this work), which lack the H2AX binding domain. On the contrary, tAIF and AIFsh, which possess both the H2AX and CypA binding sites, are active apoptogenic proteins (as demonstrated in this manuscript Figures 7, 8, and Supplementary Figure 6). Although a more detailed molecular biology study (out of the scope of the present manuscript) should be developed to improve the peptides tested in our cell free system (e.g. development of new penetrating peptides that validate these results in a cellular system), we think that our work could provide a new point of view in the activation of programmed cell death in tumor resistant cells by targeting the caspase-independent PCD pathway.
These additional data on AIF are presented in the new Supplementary Figure 10 and are explained in the Discussion section of our manuscript (page 13, paragraph 2, lines 21-27 and page 14, paragraph 1, lines 1-6). The Discussion and Materials and Methods sections have been modified accordingly (page 14, paragraph 3 and page 17 "Recombinant proteins and peptides").

Other comments:
i) Full length AIF should be shown in addition to tAIF in all figures.
As requested by this reviewer, we have included data on full length AIF and tAIF in nearly all Figures describing the function of AIF in programmed necrosis: Figures 1, 2, 6, and Supplementary Figure 7 (see below). Concerning Figures 7 and 8, it is important to remark that the use of full length AIF in a cell free in vitro system has been previously described (e.g., Delettre et al. J Biol Chem 2006, 281, 6413-6427). Thus, we have decided to evaluate two other AIF forms: AIFsh and tAIF. Indeed, the inclusion of these nuclear forms of AIF in our in vitro study seems to be more related to the present work. Finally, a detailed description of the AIF cleavage into tAIF has been incorporated into the Introduction section (including the references that demonstrate that AIF is cleaved in a caspase-independent manner) (page 3, paragraph 2).

More precisely:
(a) In Figure 1A we depict the programmed cell death system induced by high doses of MNNG in MEFs. Here, we illustrate that AIF was cleaved into tAIF by calpains;
(b) In Figure 2B, we explain the different AIF regions, underlining the calpain cleavage site that converts AIF into tAIF;
(c) In Figure 6B, we demonstrate that high doses of MNNG-treatment induce both AIF cleavage into tAIF and time-dependent mitochondrial tAIF release (see also point iii below); and
(d) In Supplementary Figure 7, we update the AIF binding domains. This includes the newly defined H2AX binding site.
In our opinion, by including full length AIF and tAIF in four Figures and in the Introduction section we provide sufficient information to t he potential readers of our manuscript. We think that the

inclusion of AIF and tAIF in Figures not related to AIF (e.g. Figures 3, 4, and 5) could disturb the rationale used in our paper.

ii) The authors should quantify the results for PAR in Fig. 5B.

The results for PAR labeling have been quantified in line with the reviewer's proposal (new Figure 5B). We have amended the Materials and Methods section that now includes the total number of cells examined.

iii) How did the authors purify AIF from the mitochondria and the inter-mitochondrial space (IMS) in Fig. 6B? Also, the authors should show by Western blots that the cytoplasmic extracts are devoid of nuclear proteins (e.g. lamin) and vice versa.

i) To purify AIF and tAIF from the mitochondria and the inter-mitochondrial space (IMS) we used the methodology described in a previous paper from our laboratory (Yuste et al. Cell Death Differ 2005, 12, 1445-1448). As described in this work, upon atractyloside treatment, mitochondrial AIF (Mit; 62 kDa) is cleaved into a lower molecular weight tAIF in the mitochondrial intermembrane space (IMS; 57 kDa). In our current manuscript, we used this in vitro test as a positive control that illustrated the AIF cleavage into tAIF in mouse embryo fibroblasts (MEFs). In this sense, we purified mitochondria from MEFs and confirmed that, as in other cellular models, AIF could be cleaved into tAIF. Alternatively, cytosolic fractions from MEFs recovered at different times after MNNG-treatment were blotted for AIF detection. Compared to the in vitro atractyloside test and the inclusion in the Western blot of the tAIF recombinant protein (tAIFr; used as an internal molecular weight marker), we demonstrated that high doses of MNNG-treatment induce both AIF cleavage into tAIF and time-dependent mitochondrial tAIF release. This substantiates our previous results published in Moubarak et al. Mol Cell Biol 2007, 27, 4844-4862.
We have revised the Materials and Methods section and the legend of Figure 6 and introduced the requested details.
ii) The reviewer asked us to verify the purity of the cytosolic and nuclear extracts, and this has been done. We have reblotted our membranes with actin and lamin A, and included the results obtained in the new Figures 6B and 6C. This complementary Western blot assessment corroborates the high purity of our cytosolic and nuclear preparations. The legends of Figures 6B and 6C have been modified accordingly.

iv) Whenever quantification was done with imaging assays, please provide information on the total number of cells examined (Figs. 3D, 4D, 5D, 6D, 7E and 8B-C).

Figures 3D, 6D, and 8C have been revised following the referee's request. In Figures 4D, 5D, 7E, and 8B, the % of cells with activated Bax or TUNEL-positive and the % of nuclei with DNA loss were quantified by flow cytometry in total population (10,000 cells/nuclei) (see also the new version of "Flow cytometry" in the Materials and Methods section).

Acceptance letter 01 March 2010

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by the original referees 2 and 3, and I am happy to inform you that there are no further objections towards publication in The EMBO Journal. Before we proceed with formal acceptance of the study, I would only like to ask you to incorporate the minor change requested in point 1 of referee 3's comments on the revision (see below) - I will leave it up to you whether or not you would also like to add an additional schematic diagram as suggested in his/her second point. In any case, please send us any modified file(s) simply via email at your earliest convenience - we will then replace them in the online tracking system and should then be able to swiftly proceed with formal acceptance and publication of your study.

Yours sincerely,

Editor
The EMBO Journal

Referee 3 (comments to authors):

The revised manuscript by Artus et al is much improved. Overall, the authors did a respectable job addressing the issues and concerns raised by the reviewers. The following minor changes will further improve the clarity of the revised manuscript.

1. AIF-PBD mut in Fig. 2 was referred to as AIF543-559 mut in the text (p. 6). It will be less confusing to keep the nomenclature consistent throughout.

2. It is not entirely clear what the mutants AIFsh2, tAIF Pro-rich Delta and AIFsh Pro-rich Delta are. A diagram similar to that used in Fig. 2B will be useful to guide the readers about the specific mutants used in these experiments.

Additional correspondence (author) 01 March 2010

Thank you very much for your positive answer. We were very pleased to learn that the referees found our paper suitable for publication in The EMBO Journal.

Concerning the comments of the referee #3, please find hereafter our response:

1. AIF-PBD mut in Fig. 2 was referred to as AIF543-559 mut in the text (p. 6). It will be less confusing to keep the nomenclature consistent throughout.

In agreement with this reviewer, we have amended page 6 of the Results section, which now keep the nomenclature consistent with Figure 2: ^{3}AIF PBD mut^{2}.

2. It is not entirely clear what the mutants AIFsh2, tAIF Pro-rich Delta and AIFsh Pro-rich Delta are. A diagram similar to that used in Fig. 2B will be useful to guide the readers about the specific mutants used in these experiments.

Mutants AIFsh2, tAIF Pro-rich Delta, and AIFsh Pro-rich Delta are now described in the Materials and Methods section ^{3}Recombinant proteins and peptides^{3} (page 17).

A modified manuscript text file (Microsoft word and pdf formats), which included the changes suggested by this referee, is attached.

We thank you for your assistance and interest regarding this manuscript.

