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ATM activates the pentose phosphate pathway promoting antioxidant defence and DNA repair

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Review timeline:

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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 10 September 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal, and please excuse the slight delay in soliciting referee reports and getting back to you with a decision in this summer vacation period. We have now finally received the reports of three expert reviewers, which you will find enclosed below. I am afraid to have to communicate that these reports do not offer strong support for publication in The EMBO Journal. Despite the potentially interesting link between ATM and the metabolic pathway upstream of NADPH generation and the antioxidant response, all referees raise a number of substantive points that clearly preclude publication in The EMBO Journal at this stage, regarding the depth of the mechanistic analysis, the demonstration of the functional importance, as well as the potential patho-physiological relevance of this link. I realize that the latter (decisively determining the relevance for A-T-associated neurodegenerative phenotypes) may well be beyond the scope of the present submission, although it would nevertheless need to be discussed more carefully also in light of diverse results in the literature as indicated by the referees. What should however be well within the scope of the study is a considerably more decisive and comprehensive demonstration of the importance of ATM-mediated PPP regulation, especially regarding effects on ROS production, nucleotide synthesis, and DNA repair in a statistically significant manner. At the same time, more conclusive data on the mechanistic link between ATM and G6PD would also be essential, regarding their interaction within intact cells, the nature of ATM regulation of Hsp27 (phosphorylation?), and the importance of Hsp27 through a knockdown experiment, to test its impact on ATM-mediated PPP activation and ATM-dependent DNA repair. Given the extent and weight of these major issues, I am afraid we have to consider the study still too premature to be a good candidate for an EMBO J paper, and therefore do not see ourselves in the position to invite a revision of the study at the current stage. That is not to say that I would exclude the possibility of looking at a significantly extended new submission on this topic once more in the future, but it is only fair to say that such a resubmission would then have to be judged on the basis of the available literature at that point, and only be sent to the back to the reviewers if we thought that at least the overriding concerns as detailed above had

been conclusively addressed.

I am very sorry that I cannot be more positive on this occasion, but for now I hope that you will in any case find the comments and suggestions of our referees useful.

With best regards,

Editor
The EMBO Journal

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REFEREE REPORTS:

In this study the authors present data indicating that radiation activates glucose-6-phosphate dehydrogenase (G6PD) and that this activation is ATM dependent. Heat shock protein 27 (Hsp27) is also required for G6PD activation. They show this initially using xenopus cell free extract and then in cells from normal and A-T individuals. Overall, the biochemical data presented makes the case for ATM-dependent radiation induced G6PD activation. While these studies are of some interest, they are limited to xenopus extracts and cell lines. Given the nature of the work, and the suggestion that the data is relevant for understanding the A-T phenotype, it would be useful to look at the link between ATM and G6PD in an in vivo setting.

Additionally, there are individuals who have glucose-6-phosphate dehydrogenase deficiency (an X-linked recessive hereditary disease), and these people are at risk of hemolytic anemia particularly after oxidative stress. However, CNS involvement is not linked to this disease, and they also don't appear to show the other clinical features of DNA repair deficiency diseases. Thus, the link between the neurological features of A-T, altered G6PD activity and DNA damage isn't strong.

The statement (rationale) in the abstract (line 5) that DNA damage explains much of the A-T phenotype, but less so the neurodegeneration is misleading. This is because there are many inherited human syndromes that result from mutations of specific DNA damage processing enzymes that are characterized by neurological problems similar to A-T. Therefore, these different human diseases suggest that problems in responding to DNA damage certainly can explain the neurological symptoms.

Other comments:

The authors propose that in response to DNA breaks (IR) that ATM stimulates G6PD activity in a manner involving hsp27. Given that ATM is nuclear and G6PD/Hsp27 are cytosolic-how do the authors suggest this nuclear/cyosolic interface is happening?

The lack of 14C incorporation in the A-T line after radiation is striking (fig 4) and should be done in multiple A-T lines. Also, no error bars are present in figure.

Unfortunately, based on the data presented for the DNA repair studies (figures 5 & 6) its not clear there is a meaningful repair deficit. The authors need additional approaches/measurements of DNA repair to substantiate their claims. They should also include controls (i.e. compare G6PD deficiency with knockdowns and depletion of known repair factors). Also, why are the basal levels of DNA damage in the human fibroblasts so high?

Referee #2 (Remarks to the Author):

In this manuscript, the authors demonstrated that ATM activates G6PD and NADPH production. They also showed that ATM stimulates the binding of G6PD with Hsp27 and that Hsp27 enhances G6PD activity in vitro. Furthermore, they showed that G6PD deficient cells are unable to repair DSB using comet assay. From these results, they suggested that ATM protect cells from ROS accumulation by stimulating NADPH production and the synthesis of nucleotides required for the

repair of DSB.
I think that the authors' results are novel and interesting. However, several problems are still not to be elucidated. My specific comments are as follows.
1) In some graphs (Figs. 1CEHI, Fig 3A, Fig4 and Fig. S1), error bars are missing. Are these experiments statistically significant? The authors should show the number of experiments and statistical analysis.
2) In Fig. 2D, the authors showed that Hsp27 enhances G6PD activity. However, this issue is based on the results using in vitro assay. To consolidate this, the authors should analyze the role of Hsp27 using Hsp27 knockdown cells by RNAi.
3) The authors suggested that ATM protect cells from ROS accumulation by stimulating NADPH production; however, this issue is not based on experimental results. Therefore, the author should analyze ROS production by DSB, and also analyze the effect of ATM and G6PD knockdown.
4) Figs. 5 and 6, the authors only analyzed the repair of DSB using comet assay. However, it is still not clear whether this effect is only caused by inhibition of the DSB repair or by induction of apoptosis. Therefore, they should show this effect using the other method.

Referee #3 (Remarks to the Author):

The use of phosphoproteomics to investigate protein phosphorylation in response to different stress stimuli suggest that ATM kinase has hundreds of substrates that are involved in diverse pathways in the cell. The more obvious candidates are those involved in the stress response to the presence of DNA double strand breaks (DSB). While a defective response to DNA DSB explains some of the phenotype in ataxia-telangiectasia (A-T) it has not been possible to determine how a failure to repair such breaks might account for the neurodegeneration which is central to this disorder. Over the years the existence of a state of oxidative stress in A-T cells and in the brains of Atm deficient mouse models point to this a candidate for the onset of neurodegeneration in A-T. The present submission investigates a possible link between ATM and the pentose phosphate pathway which is responsible for NADPH generation. Part of the rationale for this is a series of observations, referred to above, that A-T is characterised by the build-up of oxidative stress. Specifically the results showing that pyridine nucleotides are reduced in the cerebellum of Atm deficient mice and that this is progressive. The reference to a glutathione re-synthesis defect (Meredith and Dodsun 1987) is more contentious in supporting the hypothesis of an ATM/PPP link since there are other data to the contrary. Nevertheless the study described here is carefully carried out for the most part and evidence is provided to link ATM to glucose 6-phosphate dehydrogenase (G6PD) activity, a reduced cellular environment and DNA repair.
Specific Comments:
1. The Xenopus egg extract approach has been an important resource for assisting in our understanding of ATM and other DNA damage response proteins. Its use here is well supported by previous data with ATM in this system and it is evident that new important information on ATM emerges here.
2. The data in Fig 1 are generally convincing for the specific detection of G6PD activity and its induction by DNA DSB. However, several of the subsections are poorly represented in the figure and not explained sufficiently in the Legend. Part 1B - The assumption is that G6PD is the top band but no indication of size etc is provided on this cropped blot. A more explanatory blot is required. Part D reveals a series of bands which I assume from the legend are ATM/ATR substrates. What are they? Phosphorylated forms? More details on Figure and in Legend required. Part E, key to symbols missing. Part H - It is difficult to determine what activity levels are being measured for eluates. How does this activity compare to data in Fig 1C for example? It is claimed that the data in Fig 1, E,F suggests that ATM strongly provides NADPH (NAPDH!!) production. It looks like 1-6 fold increase. How significant is this?
3. The suppression of the interaction between G6PD and Hsp27 by ATMi suggests that ATM plays a role in phosphorylating one of these proteins. There appears to be a "tongue-in-cheek" statement on page 7 suggesting this "might involve post-translational modifications of Hsp27. This may be known by now but should be checked ie does ATM activation lead to phosphorylation of Hsp27 either directly or indirectly.
4. The data in Fig 2D demonstrate a direct effect of Hsp27 on G6PD activity. While this is clearly shown in vitro the question that arises is how this activation relates to what might happen in the cell. A range of 40-120 ng/ml of Hsp27 is employed. How does this relate to physiological concentrations expected?

5. The comparison of G6PD activity in normal human cells versus A-T cell lines showed a reduced IR response in A-T cells (Fig 3B). These data point to the involvement of a second system. Might this be ATR or another PIKK? This should be testable in the Xenopus system.
6. The effect of depletion of G6PD on DNA repair in human cells is rather small (Fig 6B). Is this in keeping with the increase in radiosensitivity in cells depleted of G6PD. It would be nice to see the radiosensitization data.
7. As referred to in the opening comments the Meredith and Dodson observations that glutathione re-synthesis was defective in A-T is only one side of the story. The authors should read later correspondence in Cancer Research 48: 5374-5376 (1987) which describes data with the same cells where there was a failure to observe any differences.
8. The authors need to go over the English presentation in this manuscript more carefully. There are many examples of wrong tense, poor grammar etc.

Resubmission - authors' response 27 October 2010

We are submitting a revised version of our manuscript entitled "ATM activates the pentose phosphate pathway promoting antioxidant defence and DNA repair"
Briefly, in this manuscript we show that ATM regulates the pentose phosphate pathway (PPP) through Hsp27 mediated stimulation of the Glucose-6-Phosphate Dehydrogenase (G6PD), the limiting enzyme of the PPP responsible for the production of NADPH, an essential anti-oxidant cofactor.
We were pleased that you and the referees found our manuscript novel and interesting in the previous round of revision. We have worked very hard to answer referee comments. We now believe that we have fully addressed all the critiques raised providing a more decisive and comprehensive demonstration of the importance of ATM-mediated PPP regulation.
As requested by you and the referees we have included statistical significance for all the experiments shown. At the same time, we present more conclusive data on the mechanistic link between ATM and G6PD by showing that ATM promotes Hsp27 phosphorylation and binding to G6PD stimulating its activity. Importantly, we demonstrated that Hsp27 is required for ATM mediated activation of G6PD through a knockdown experiment. Furthermore, we tested the impact of this pathway on DNA repair using different assays in addition to what already presented.
Overall these new results confirm and reinforce previous findings showing that ATM is a key player in the regulation of the PPP and that this might be an important factor to consider when analysing the underlying causes of the clinical manifestations of ATM deficiency in human cells.
In light of these important new results we would like to ask you to reconsider our manuscript.
I have included a response to referee comments in this cover letter. Your letter and referee comments are attached.

I thank you in advance for your consideration.

Referee #1 (Remarks to the Author):
In this study the authors present data indicating that radiation activates glucose-6-phosphate dehydrogenase (G6PD) and that this activation is ATM dependent. Heat shock protein 27 (Hsp27) is also required for G6PD activation. They show this initially using xenopus cell free extract and then in cells from normal and A-T individuals. Overall, the biochemical data presented makes the case for ATM-dependent radiation induced G6PD activation. While these studies are of some interest, they are limited to xenopus extracts and cell lines. Given the nature of the work, and the suggestion that the data is relevant for understanding the A-T phenotype, it would be useful to look at the link between ATM and G6PD in an in vivo setting.

Our response:
We would like to thank this referee for the insightful comments, which greatly helped to improve our manuscript. We would like to point our studies are based on both primary cell lines as well as Xenopus egg extract and that in both systems we have found similar results. This suggests that the pathway that we uncovered is very conserved. We agree about the requirement for a more in vivo setting to link this pathway to the neurodegeneration found in A-T. However, we feel that this goes

beyond the scope of this initial characterization. We have, therefore, more carefully discussed the impact that our observations might have on A-T, taking in consideration that this mechanism is unlikely to be the only one responsible for the A-T phenotype.

Additionally, there are individuals who have glucose-6-phosphate dehydrogenase deficiency (an Xlinked recessive hereditary disease), and these people are at risk of haemolytic anaemia particularly after oxidative stress. However, CNS involvement is not linked to this disease, and they also don't appear to show the other clinical features of DNA repair deficiency diseases. Thus, the link between the neurological features of A-T, altered G6PD activity and DNA damage isn't strong.

Our response:
The phenotype due to lack of functional ATM is complex due to variety of pathways controlled by ATM. Therefore it is unlikely that the control of G6PD by ATM is entirely responsible for the defects observed in A-T patients. G6PD deficient cells have a functional ATM gene. Therefore, while the simple lack of G6PD is not sufficient to induce CNS defects, ATM deficiency and the consequent lack of ATM mediated stimulated G6PD activity might produce additive effects not present in isolated G6PD deficiency.
It is also possible that the inborn deficiency of G6PD induces early systemic changes in alternative pathways that compensate for the lack of G6PD activity. As pointed out by this referee the defect in G6PD leads mainly to haemolytic anaemia, which affects just erythrocytes and not other cells. Production of NADPH is a complex process and is ensured by more that one enzyme such as the NADP+-linked isocitrate dehydrogenase, the 6-phosphogluconate dehydrogenase and the malic enzyme, whereas the PPP in erythrocytes is, in essence, the only pathway for these cells to produce NADPH. Indeed, although the PPP is considered to be the major source of NADPH in the cytoplasm, genetic defects in G6PD, which catalyzes the first step in the PPP, do not affect sterol or fatty acid metabolism, which requires significant amounts of NADPH in humans. This result suggests that the contribution of other enzymes to NADPH production in cells different from erythrocytes may be significant. At this stage we cannot exclude that these other enzymes are also under the direct control of ATM.

The statement (rationale) in the abstract (line 5) that DNA damage explains much of the A-T phenotype, but less so the neurodegeneration is misleading. This is because there are many inherited human syndromes that result from mutations of specific DNA damage processing enzymes that are characterized by neurological problems similar to A-T. Therefore, these different human diseases suggest that problems in responding to DNA damage certainly can explain the neurological symptoms.

Our response:
We did not intend saying that deficiency in DNA repair does not account for defects in the CNS. Accordingly, we have now modified this statement pointing out that defects in response to ROS together with DNA repair impairment can both contribute CNS defects. Consistent with a defect in ROS response of A-T cells we have now cited and discussed the recent work from Dr Tanya Paull group (ATM activation by oxidative stress. Guo Z, Kozlov S, Lavin MF, Person MD, Paull TT. Science. 2010 Oct 22;330(6003):517-21) showing that ATM is a sensor of ROS and that a mutant ATM protein, which does not respond to ROS is responsible for an A-T variant disease in which CNS involvement is present.

Other comments:
The authors propose that in response to DNA breaks (IR) that ATM stimulates G6PD activity in a manner involving hsp27. Given that ATM is nuclear and G6PD/Hsp27 are cytosolic-how do the authors suggest this nuclear/cyosolic interface is happening?

Our response:
We now show that HSP27, which is mainly cytoplasmic, is phosphorylated in an ATM dependent manner on serine 78, which is substrate of p38-MK2 (Figure 2e). It has recently been reported that the p38-MK2 pathway is activated by DNA damage and that P38-MK2 translocates from nuclei to cytoplasm after being activated by ATM (DNA damage activates a spatially distinct late

cytoplasmic cell-cycle checkpoint network controlled by MK2-mediated RNA stabilization. Reinhardt HC et al Mol Cell. 2010 Oct 8;40(1):34-49). Consistent with this we have verified that phosphorylation Hsp27 serine 78 upon IR can be inhibited by ATM and p38 inhibitors. Therefore, it is entirely possible that ATM dependent control of cytoplasmic Hsp27 is mediated by the cytoplasmic branch of p38-MK2 pathway activated by ATM.

The lack of 14C incorporation in the A-T line after radiation is striking (fig 4) and should be done in multiple A-T lines. Also, no error bars are present in figure.

Our response:
We have now included statistical significance for this and other experiments shown. We have performed similar experiments in multiple cell lines confirming these findings. Data obtained in different cell lines can be made available to this referee.

Unfortunately, based on the data presented for the DNA repair studies (figures 5 & 6) itís not clear there is a meaningful repair deficit. The authors need additional approaches/measurements of DNA repair to substantiate their claims. They should also include controls (i.e. compare G6PD deficiency with knockdowns and depletion of known repair factors). Also, why are the basal levels of DNA damage in the human fibroblasts so high?

Our response:
We have now presented data about the effects of G6PD deficiency on DNA repair using two different assays based on comet and phospho-H2AX detection (Figure 6) showing that there is a repair deficit similar to the one present in ATM deficient cells. We have also included data relative to inhibition of DSB repair factor DNA-PK for comparison.

Referee #2 (Remarks to the Author):
In this manuscript, the authors demonstrated that ATM activates G6PD and NADPH production. They also showed that ATM stimulates the binding of G6PD with Hsp27 and that Hsp27 enhances G6PD activity in vitro. Furthermore, they showed that G6PD deficient cells are unable to repair DSB using comet assay. From these results, they suggested that ATM protect cells from ROS accumulation by stimulating NADPH production and the synthesis of nucleotides required for the repair of DSB.
I think that the authors' results are novel and interesting. However, several problems are still not to be elucidated. My specific comments are as follows.
1) In some graphs (Figs. 1CEHI, Fig 3A, Fig4 and Fig. S1), error bars are missing. Are these experiments statistically significant? The authors should show the number of experiments and statistical analysis.

Our response:
We thank this referee for his/her insightful comments that helped to improve our manuscript. We ha originally stated that the data presented were representative results from multiple experiments. We have now included statistical significance for all the data shown.

2) In Fig. 2D, the authors showed that Hsp27 enhances G6PD activity. However, this issue is based on the results using in vitro assay. To consolidate this, the authors should analyze the role of Hsp27 using Hsp27 knockdown cells by RNAi.

Our response:
We have now successfully performed this experiment, which shows that knockdown of Hsp27 abolishes ATM mediated increase of G6PD activity (Figure 4).

3) The authors suggested that ATM protect cells from ROS accumulation by stimulating NADPH production; however, this issue is not based on experimental results. Therefore, the author should analyze ROS production by DSB, and also analyze the effect of ATM and G6PD knockdown.

Our response:
Increased production of ROS has been previously documented in ATM deficient cells. We have now addressed the contribution of G6PD in the response to ROS production following administration of ionizing radiation. We now show that G6PD inhibition leads to a direct increase in ROS accumulation following administration of DNA damaging agents. This indicates that G6PD is involved in suppressing accumulation of ROS (Figure S7).

4) Figs. 5 and 6, the authors only analyzed the repair of DSB using comet assay. However, it is still not clear whether this effect is only caused by inhibition of the DSB repair or by induction of apoptosis. Therefore, they should show this effect using the other method.

Our response:
We have included an additional assay based on the detection of H2AX phosphorylation as readout of DSB repair (Figure 6C and 6D). We have also excluded the activation of the apoptotic pathway by monitoring Caspase 3 activation (Figure S5).

Referee #3 (Remarks to the Author):
The use of phosphoproteomics to investigate protein phosphorylation in response to different stress stimuli suggest that ATM kinase has hundreds of substrates that are involved in diverse pathways in the cell. The more obvious candidates are those involved in the stress response to the presence of DNA double strand breaks (DSB). While a defective response to DNA DSB explains some of the phenotype in ataxia-telangiectasia (A-T) it has not been possible to determine how a failure to repair such breaks might account for the neurodegeneration which is central to this disorder.
Over the years the existence of a state of oxidative stress in A-T cells and in the brains of Atm deficient mouse models point to this a candidate for the onset of neurodegeneration in A-T. The present submission investigates a possible link between ATM and the pentose phosphate pathway, which is responsible for NADPH generation. Part of the rationale for this is a series of observations, referred to above, that A-T is characterised by the build-up of oxidative stress. Specifically the results showing that pyridine nucleotides are reduced in the cerebellum of Atm deficient mice and that this is progressive. The reference to a glutathione re-synthesis defect (Meredith and Dodsun 1987) is more contentious in supporting the hypothesis of an ATM/PPP link since there are other data to the contrary.
Nevertheless the study described here is carefully carried out for the most part and evidence is provided to link ATM to glucose 6-phosphate dehydrogenase (G6PD) activity, a reduced cellular environment and DNA repair.

Our response:
We thank this referee for the positive and insightful critiques that greatly helped to improve our manuscript. We have now addressed all the critiques raised and we have discussed the interesting literature mentioned by this referee such as the work from Meredith and Dodson, which in principle is in agreement with our findings.

Specific Comments:
1. The Xenopus egg extract approach has been an important resource for assisting in our understanding of ATM and other DNA damage response proteins. Its use here is well supported by previous data with ATM in this system and it is evident that new important information on ATM emerges here.

Our response:
We thank this referee for the acknowledgement of our efforts to study this novel pathway using the powerful Xenopus egg extract.

2. The data in Fig 1 are generally convincing for the specific detection of G6PD activity and its induction by DNA DSB. However, several of the subsections are poorly represented in the figure and not explained sufficiently in the Legend. Part 1B - The assumption is that G6PD is the top band

but no indication of size etc is provided on this cropped blot. A more explanatory blot is required. Part D reveals a series of bands which I assume from the legend are ATM/ATR substrates. What are they? Phosphorylated forms? More details on Figure and in Legend required. Part E, key to symbols
missing. Part H - It is difficult to determine what activity levels are being measured for eluates. How does this activity compare to data in Fig 1C for example? It is claimed that the data in Fig 1, E,F suggests that ATM strongly provides NADPH (NAPDH!!) production. It looks like 1-6 fold increase. How significant is this?

Our response:
We have now improved the presentation of the panels in Figure 1 according to referee suggestion. We have included a novel western showing G6PD depletion and indicated the size marker in panel 1B. We now show a western with anti phospho ATM serine 1981 in panel 1D as better indicator of ATM activation instead of ATM/ATR substrates. The activity of G6PD in the eluates shown in Figure 1H is comparable to the one obtained from extracts as the assay as been calibrated for a specific amount of protein to give similar levels of activity. We have also included statistical significance for all the experiments presented, which shows that the data are extremely reproducible.

3. The suppression of the interaction between G6PD and Hsp27 by ATMi suggests that ATM plays a role in phosphorylating one of these proteins. There appears to be a "tongue-in-cheek" statement on page 7 suggesting this "might involve post-translational modifications of Hsp27. This may be known by now but should be checked ie does ATM activation lead to phosphorylation of Hsp27 either directly or indirectly.

Our response:
We have now tested Hsp27 phosphorylation and shown that ATM promotes Hsp27 phosphorylation on serine 78, which is a known phosphosite for p38-MK2 (Figure 2e). Therefore, we propose that ATM promotes p38-MK2 dependent phosphorylation of Hsp27. This is consistent with cytoplasmic p38-MK2 pathway being activated by nuclear ATM as recently shown (DNA damage activates a spatially distinct late cytoplasmic cell-cycle checkpoint network controlled by MK2-mediated RNA stabilization. Reinhardt HC et al Mol Cell. 2010 Oct 8;40(1):34-4)

4. The data in Fig 2D demonstrate a direct effect of Hsp27 on G6PD activity. While this is clearly shown in vitro the question that arises is how this activation relates to what might happen in the cell. A range of 40-120 ng/ml of Hsp27 is employed. How does this relate to physiological concentrations expected?

Our response:
We have measured the concentration of Hsp27 and related it to the endogenous level (Figure S2). The amounts used here to obtain G6PD stimulation are lower than the endogenous levels of Hsp27. This is expected given the multiplicity of Hsp27 targets in the cell.
5. The comparison of G6PD activity in normal human cells versus A-T cell lines showed a reduced IR response in A-T cells (Fig 3B). These data point to the involvement of a second system. Might this be ATR or another PIKK? This should be testable in the Xenopus system.

Our response:
This is an interesting point that unfortunately we could not address in the Xenopus system due to the lack of ATR depleting antibody. In addition there are no chemicals that are specific just for ATR.
Therefore, we could not perform this experiment.

6. The effect of depletion of G6PD on DNA repair in human cells is rather small (Fig 6B). Is this in keeping with the increase in radiosensitivity in cells depleted of G6PD. It would be nice to see the radiosensitization data.

Our response:

Unfortunately depletion of G6PD results in poor long-term survival. Therefore, the effects on the radiosensitivity are difficult to measure. In any case we have included these data in figure S3, where it can be noted that IR treatment further impairs survival of G6PD knockdown cells.

7. As referred to in the opening comments the Meredith and Dodson observations that glutathione re-synthesis was defective in A-T is only one side of the story. The authors should read later correspondence in Cancer Research 48: 5374-5376 (1987) which describes data with the same cells where there was a failure to observe any differences.

Our response:
We have cited and discussed studies showing a correlation between redox state, measured as reduced levels of glutahtione and severity of the A-T phenotype, measured as cerebellar atrophy (In ataxia-teleangiectasia betamethasone response is inversely correlated to cerebellar atrophy and directly to antioxidative capacity. Russo I, Cosentino C, Del Giudice E, Broccoletti T, Amorosi S, Cirillo E, Aloj G, Fusco A, Costanzo V, Pignata C.) This report confirmed earlier findings about defects in resynthesis of glutathione in A-T cells (Meredith and Dodson 1987). At this stage we are unable to establish the reasons why Meredith and Dodson observations were not reproduced. In any case we should point out that unlike these studies our experiments did not directly address the levels of glutathione in A-T cells but the levels of NADPH. Therefore, we cannot dispute or prove Meredith and Dodson observations although these are in agreement with others and ours findings.

8. *The authors need to go over the English presentation in this manuscript more carefully. There are many examples of wrong tense, poor grammar etc.*

Our response:
We have proof read the article. Unfortunately, a previous un-proofed version was submitted by mistake.

Acceptance letter 23 November 2010

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by the three original referees (see comments below). I am happy to inform you that in light of these comments we consider the manuscript now suitable for publication in The EMBO Journal. Some reservations regarding the immediate pathophysiological significance of your results for further understanding A-T remain remain but as discussed earlier this may be reaching beyond the scope of the current study. Importantly, the more concrete concerns regarding the conclusiveness of the results and the depth of insight into the new regulatory link appear to have been satisfactorily addressed and I therefore do not see any further objections against acceptance.

Before we will able to send you a formal letter of acceptance, there are just two minor things I need to ask you for: a 'conflict of interest' statement, and an 'author contribution' statement, both to be included at the end of the manuscript text. To expedite this, you may simply send them to us in the body of an email, from which we can easily copy them into the manuscript text file; alternatively you may send as a new text document including these two statements.

After that, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

Yours sincerely,

Editor
The EMBO Journal

Referee #1

(Remarks to the Author)

The additions of data and text changes to the manuscript have strengthened the data set and improved accuracy and readability. Nonetheless, the concerns outlined in the review of the original submission remain, particularly regarding the actual significance of the finding and the lack of physiological context for further understanding ataxia telangiectasia. The DNA repair data is also still not particularly convincing and doesn't really make a clear case for a repair defect after G6PD inhibition.

Referee #2

(Remarks to the Author)
I think that this revised manuscript is now suitable for publication in the EMBO Journal.

Referee #3

(Remarks to the Author)
The authors have addressed all the issues raised by the 3 reviewers.They have adequately dealt with all the points that I raised.Specifically they have improved the presentation of Figure 1 and presented statistical significance where appropriate.The issue of error bars was also raised by reviewers 1 and 2 and both points appear to be adequately addressed.Reviewer 1 was concerned about the evidence for a DNA repair defect.This has been fixed by the inclusion of a new assay and evidence that the deficit is comparable to that in a-T cells.The new data appear in Fig 6.
One issue that was raised by two of the reviewers was the difficulty of ATM stimulating G6PD through hsp27 given that ATM is in the nucleus and hsp27 in the cytoplasm and whether this was achieved by a specific phosphorylation on hsp27.The authors have idenntified that site as S78 on hasp27 and presented the data in Fig2E.They also rationalise how it is the signalling occurs through p38-MK2.They also showed that knockdown of hasp27 prevents the stimulation of G6PD activity by ATM.Evidence for increased ROS production is also provided.
In summary the authors have addressed all the issues raised and carried out additional experimnts as requested.I recommend that the manuscrpt be accepted for publication in EMBO J.

