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An indirect role for ASPP1 in limiting p53-dependent p21 expression and cellular senescence

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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 05 September 2011

Thank you very much for submitting your paper reporting a novel, indirect role for cytoplasmic ASPP1 in regulating p53-dependent p21 induction for consideration to The EMBO Journal editorial office. As you will see from the enclosed comments, all three scientists appreciate this new mechanistic aspect, though insist on further clarifications that should include some experimental work to further elucidate functional ASPP1/YAP interaction. As the comments are relatively explicit in what is expected, I am happy to invite submission of a carefully amended version for final assessment.
Please be reminded that it is EMBO Journal policy to allow a single round of revisions only and that the ultimate decision on acceptance depends on the content and strength of the revised manuscript.

I remain with best regards and look very much forward receiving your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

In this manuscript, Vigneron AM et al. report indirect regulation of one p53 responsive target, p21, by cytoplasmic ASPP1. They found that co-overexpression of ASPP1 and YAP selectively regulates

the expression of p53 target genes, including p21, Bax, Puma, et al. Also ASPP1-caused nuclear accumulation of YAP led to the suppression of p53-dependent p21 induction and cell cycle arrest via inhibition of LATS2's transcription by YAP. Consistently, depletion of ASPP1 or of YAP enhanced DNA replication inhibitor-induced senescence. This study is potentially interesting and documents a novel function of cytoplasmic ASPP1 to inhibit p53 transcriptional activity, which is opposite to the well-known role of nuclear ASPP1 in enhancing p53 transcriptional activity. The manuscript is well prepared and written. However, addressing the following concerns would help them improve their manuscript.

1. In light of previous study that ASPP1 increased endogenous YAP nuclear localization, may the authors explain why overexpression of ASPP1 alone did not affect p53 transcriptional activity.
2. For Fig. 1D, may the authors describe briefly how to collect cells with high or low p21 expression.
3. The authors found that co-overexpression of ASPP and YAP repressed p53 target genes, including p21, Bax and Puma (Fig. 1C), all of which are related to cell cycle progression or cell proliferation. However, their data also show that ASPP1/YAP down-regulation has no effect on cell proliferation in p21-depleted cells at all (Fig. 2E). These two observations are incompatible.
4. The assertion that p21 induction in ASPP1 depleted cells leads to S-phase arrest may be partially due to HU treatment, as in most cases, induction of p21 causes G1 or G2-arrest. May the authors test the cell cycle progression under normal culturing condition or with Doxorubicin treatment?
5. May the authors show overexpression of YAP or ASPP1 alone, if not, co-overexpression of YAP and ASPP1, can suppress LATS2 mRNA expression?

Referee #2:

This manuscript describes an interesting and novel function of cytoplasmic ASPP1 in regulating cell cycle progression. It is proposed that cytoplasmic ASPP1 functions together with YAP to repress LATS2 expression. As a result, ASPP1 is able to indirectly prevent p53 from inducing p21's expression. A number of findings are interesting, and the majority of the data are convincing. However, clarification of the following issues is needed before the publication of this manuscript.

1) In U2OS cells, ASPP1 and YAP act synergistically to repress p21waf1 expression (figure 1C-1E). However, this synergistic effect was not seen in HCT116 cells (figure 2). Can the authors provide an explanation for this?

2) In figure 2 the authors show that the ability of ASPP1 to inhibit G1 arrest is p53 and p21 dependent. Can YAP use the same pathway to inhibit S-phase entry? This is important, as YAP is known to bind p73 and enhance its function. Can ASPP1 and YAP function together to repress p21 in p53-deficient HCT116 cells, similar to that seen in U2OS cells?

3) The finding that ASPP1 enhances YAP's ability to bind the LATS2 promoter is interesting. Nonetheless, how YAP binds the LATS2 promoter remains unclear. Is this p73 or TAZ dependent? Can ASPP1 affect LATS2 expression via the activity of p73 or TAZ?

4) When the authors looked at the effect on cellular senescence, it seems that depletion of either ASPP1 or YAP1 resulted in a similar number of senescent cells. However, when they looked at the effect of ASPP1 or YAP depletion on colony formation in response to Doxorubicin or Hydroxyurea treatment, YAP1 deletion had a more profound impact on reducing colony formation than ASPP1. A similar trend was also seen in p53-deficient HCT116 cells. It is therefore possible that, although YAP1 is predominantly able to maintain cell viability through a p53-dependent pathway, it could also use a p53-independent pathway. Once again, it is important that the authors explain or discuss the roles of the p73/YAP connection.

Minor points:
1) The Y-axis labels of figure 1G and sup figure S2 are missing and need to be included.
2) The Y-axis labels in figures 6B-D need to be clearly defined in the figure legend. Was it possible for there to be over 225,000 colonies per dish (figure 6D)?

Referee #3:

The manuscript by Vigneron and Vousden describes data supporting an indirect role for ASPP1 in p53-dependent p21 induction and senescence. It is a clearly written manuscript on a novel mechanism of p53 regulation that expands the authors' previous work. The data and rationale are explained well. Only minor criticisms exist for this manuscript.

Error bars are missing for Fig 2A and 2B.

A control siRNA for the left side of Fig 2E is missing.

Unclear why iASPP was not added to some of the experiments, such as for Fig 3 to complement the siASPP1 approach.

For Fig 5A, siASPP1 is shown to increase LATS2 mRNA, but there is no increase in LATS2 protein in Fig 5B. Please explain.

Statistical analysis for Fig 6B, 6C, and 6D are needed. Testing HCT116 p21-/- cells for Fig 6C would also be helpful.

Refs for page 7 are needed where the authors refer to "several previous studies".

The authors do not show data with p53 deleted cells, for Fig 3, so they need to put "data not shown" on page 9 at the end of the sentence "Thus effect was observed in both p53 expressing and p53 deleted cells."

A pathway schematic figure to summarize the conclusions of the manuscript would be helpful for readers who are not experts in the field.

1st Revision - Authors' Response 13 October 2011

Thank you for returning the reviewers comments on our paper "An indirect role for ASPP1 in limiting p53-dependent p21 expression and cellular senescence". We are very happy that the reviewers found our work interesting, and thank them for their suggestions for improving the study. We have now revised the paper to address these comments, including additional data and explanation.

Referee 1

1. The reviewer points out that we were unable to detect an effect of ASPP1 alone on p53 transcriptional activity in U2OS cells (Figure 1C). As shown in Figures 1A and 1B, these cells make very little (if any) endogenous YAP, and so we assume that transfection of ASPP1 alone will not be able to activate the pathway we describe. We apologize for not making this clearer, and have added a sentence on page 7 to clarify this point.
2. We have added a description of how cells with high and low p21 expression were detected in the Figure legend (Figure 1D).
3. In U2OS cells overexpressing ASPP1 and YAP, we detected a reduction in expression of a number of p53-target genes, as pointed out by the reviewer. However, the critical role of p21 was identified in HCT116 cells, where we detected only very low levels of PUMA and Bax expression in the absence of p53-activating signals (as shown in Figure 2). Consistently, we found that while p21 and MDM2 levels were controlled by basal p53 levels in unstressed cells, PUMA and Bax levels were not significantly altered by depletion of p53. We therefore believe that p21 is the major regulator of p53-dependent cell cycle arrest in these cells. We have now included these data in Supplementary Figure 3 and include an explanation of the results in the text on page 8.
4. As predicted by the reviewer, we show in Figure 2 that ASPP1 and YAP depletion can result in a G1 arrest under normal culturing conditions.

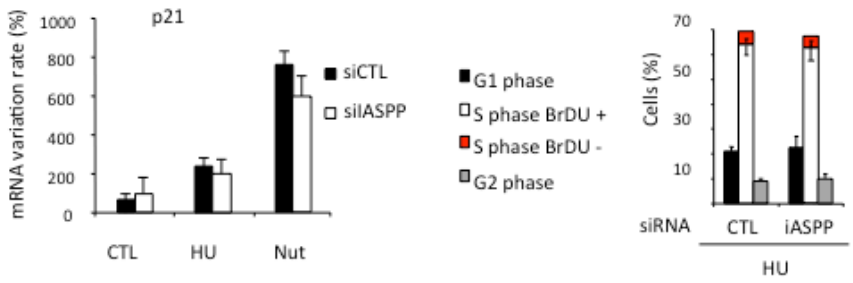
5. We have now included a Western blot showing the down-regulation of LATS2 expression following ASPP1/YAP overexpression under basal conditions and after Nutlin treatment, as predicted by the reviewer. These data are in new Figure 5C.

Referee 2

1. The referee makes a comment that is related to comment 1 from reviewer 1. While U2OS cells make very little endogenous ASPP1 or YAP (Figure 1), both of these proteins are highly expressed in HCT116 cells (as shown in Vigneron et al, G&D 2010). We therefore predict that depleting either YAP or ASPP1 would impede the activation of the pathway we describe in HCT116 cells since they are both required for the activation of the response. For the same reason, adding only ASPP1 or YAP to the U2OS cells is insufficient to induce this pathway. We are sorry for not making this clear, and have added further explanation in the text on page 8.
2. As suggested by the reviewer, we looked at the effect of ASPP1 knock down in p53 null cells, but did not see any changes in p21 levels. These data are now included in new Supplementary Figure 4 and discussed on page 9. We therefore conclude that the effects of YAP are mediated mostly through p53, not p73, in these cells.
3. Under the conditions we have tested YAP binding to the LATS promoter, we have not induced p73 expression, which is therefore very low in these cells (as seen in Vigneron et al, G&D 2010). Furthermore, YAP does not bind to the p53-binding site in the promoter (potentially shared with p73). We therefore do not believe that the effects of ASPP1 are mediated via p73 in this experimental system. Our data suggest that YAP binds the LATS2 promoter through another transcription factor such as TEAD, one of the principal targets of YAP. We have included a discussion of this point in the text on page 12.
4. We have found previously that genotoxic stress leads to the induction of p73, and we assume that the difference in senescence and colony number reflects the induction of apoptosis through p73 and Bim-dependent mechanisms. We have now clarified this in the text on page 14.
5. The labeling of the graphs has been clarified. Colonies were counted in large dishes by microscope, counting 20 random field and extrapolating to the whole dish, the details have been added to the materials and methods section.

Referee 3

1. Error bars have been included in Figures 2A and B.
2. The siRNA control for this experiment is the same as for Figure 2C. We have added this to Figure 2E but noted in the legend that these are the same data as in Figure 2C.
3. We examined the effect of iASPP depletion as suggested by the reviewer, but saw no effect of iASPP siRNA, as shown below. This is not surprising, however, since HCT116 cells make very low levels of endogenous iASPP (Figure 1). We also considered using iASPP overexpression but felt that this might not be informative for the present study, since iASPP would be expected to counteract the effect of nuclear ASPP1, and we are focused in this report on the activities of cytoplasmic ASPP1.



4. The results in Figure 5B show a slight increase in LATS2 protein levels following depletion of ASPP1 under both control conditions and in HU treated cells (compare lanes 1&2, or lanes 3&4). We agree with the reviewer that this effect is modest, but we have reproducibly seen

this effect in numerous experiments, in agreement with the increase in LAST2 mRNA levels shown in Figure 5A.
5. We have added the results in p21 null cells to Figure 6C.
6. We have added p-values to the data in Figures 6 B. In Figures 6 C and D the differences were did not reach significance ($p>0.05$); we have added this information to the Figure legend.
7. We have added the references on page 7.
8. We have indicated that the cell cycle dependent modulation of ASPP1 expression in p53 null cells is data not shown.
9. We have included a model as supplementary Figure S8 .

We would like to thank the reviewers for their suggestions and believe that addressing the comments has greatly improved the paper. I hope you will now find it suitable for publication in the EMBO Journal.