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Asf1b, the necessary Asf1 isoform for proliferation, is predictive of outcome in breast cancer

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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 06 August 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has been evaluated by three referees and I enclose their reports below. They currently provide mixed recommendations and require further experimental work to make the study suitable for The EMBO Journal.

From these comments the main interest in the study comes from the isoform specific analysis of Asf1b, with referee #1 and #2 being more positive than referee #3. However, based in the current data the referees are not completely convinced of a specific role in proliferation for Asf1b and require that a panel of breast cancer cell lines are analysed for Asf1b expression and the effect of Asf1b depletion demonstrated on proliferation of these cell lines and colony forming capacity. In relation to this, the referees raise concerns with the depletion experiments being performed in osteosarcoma cell lines and require that these experiments be performed in breast cancer cells. From my point of view I do not necessarily think that the genome-wide expression analysis be repeated, but the Asf1b dependent expression of S-phase linked cells should be confirmed in breast cancer cell lines. Referee #2 also requires some insight into how depletion of Asf1b contributes to proliferation defects. Both referee #2 and #3 (independent of his/her report) question if the sample size is sufficient to make strong statements on the prognostic predictive conclusions, which I would appreciate if you could discuss. Given the interest in the study should you be able to address these issues, we would be wiling to consider a revised manuscript.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This manuscript describes the physiological differences between two isoforms of Anti-silencing function 1 (Asf1), Asf1a and Asf1b. The authors report that: I) human Asf1b shows proliferation-dependent expression in cycling and tumoral cells; II) deletion of Asf1b compromises proliferation and leads to aberrant nuclear structure; III) these properties of Asf1b are unparalleled by Asf1a. Further, based on the higher expression level of Asf1b mRNA in breast cancer cells than normal cells, the authors point out that Asf1b could become an effective proliferation marker for breast cancer diagnosis and prognosis. This claim is verified using tissue samples derived from early stage breast tumors. As a whole, the relationships between Asf1b and proliferation, and the effectiveness of Asf1b as a proliferation marker are sufficiently investigated in several types of cells and tissue samples. However, the claim that Asf1b is necessary for proliferation remains debatable. Major and minor comments to help to improve the manuscript are as follows.

Major comments:

1) (page 2) In "Abstract" section, although the authors describe basic and applied aspects on Asf1b, it is quite confusing which aspect(s) the authors intend to mainly claim. If their intention is to claim both aspects equally, rephrasing in "Abstract" like "we elucidated the functional importance of Asf1b about basic and applied aspects. As the former aspect, we... " would improve the clarity of authors' claims. Even if the authors focus on either of aspects, they should make it clear in "Abstract" section.

2) (page 3) The first paragraph in the "Introduction section" is remotely related to the main claims including the physiological roles of Asf1b in proliferation, and the effectiveness of Asf1b as a proliferation marker in breast cancer. Rewrite of the paragraph or whole constitution of "Introduction" would be of great help for the readers to understand the gist of the manuscript.

3) (page 5, paragraph 2) This paragraph is too long. To shorten this paragraph would make the manuscript more readable.

4) (Figure 2A) The authors show that tumoral and normal cells contain 25 and 13 % of cells in S phase, respectively (Supplementary Figure S4A). This result suggests the possibility that different expression levels of Asf1s between tumoral and normal cells as shown in Figure 2A do not reflect different properties of tumoral and normal cells, but indicate just the gap of cell cycle between these cell lines. In order to confirm the higher expression level of Asf1b in tumoral cells than normal cells without the effect of different cycling status, the authors should quantify the western blot results in Figure 2A like "Western blot quantification by chemiluminescence" in the "Supplementary

Materials and Methods" section.

5) (Figure 4D) In this manuscript, the claim that Asf1b is necessary for proliferation is only sustained by the colony formation assay as shown in Figure 4D. However, this assay evaluates cell viability, which includes cell death as well as proliferative capacity. Therefore, I'm concerned whether the effect of Asf1b deletion in the assay is attributed only to the perturbation of proliferation. To verify the effects of Asf1b on proliferation, the authors checked the effect of Asf1b deletion on cell cycle (Figure 3A), the localization of a DNA replication-related factor (PCNA) (Supplementary Figure S6C), the amount of γH2A.X as a marker of DNA damage (Supplementary Figure S6D). However, there is no discussion on cell death in Asf1b-deficient cells. The authors should carefully reconsider whether Asf1b is a bona fide regulator necessary for proliferation.

6) (Figures 3 and 4) One of the main claims of this manuscript is that Asf1b becomes an effective proliferation marker in breast cancer cells. While breast cancer cell lines are used in Figures 1 and 2, an osteosarcoma cell, U-2-OS cell, is used in Figures 3 and 4. To verify the claim in a consistent manner, the authors should use a breast cancer cell, MCF7 cell, for Figures 3 and 4, or explain the reason why the use of U-2-OS cell is appropriate. Even in the latter case, proliferation-dependent expression of Asf1b should be shown in U-2-OS cells as in MCF7 cells (Figure 1A).

7) (Figure 4D) The authors illustrate the reduced competence of colony formation in the cells treated with siAsf1b or siAsf1(a+b). In the manuscript, the authors described that "Surprisingly, the combined depletion of Asf1(a+b) did not give as strong an effect as Asf1b depletion alone on nuclei morphology and proliferation (Figure 4B and 4D)'(page 10, paragraph 1, lines 14-15). However in Figure 4D, there isn't distinct difference of the survival rates between cells treated with siAsf1b and siAsf1(a+b). The authors should explain how they evaluate the results.

Minor comments:

1) (page 3, paragraph 1, line 9) [Eitoku et al., Cell. Mol. Life Sci., 65, 414-444 (2008)] would also be an appropriate review article since it discusses histone chaperones as a whole.

2) (page 3, paragraph 2) The reason why the authors focus on Asf1 should be explained.

3) (page 3, paragraph 2, line 6) Although the authors note "mammals possess two Asf1 isoforms, Asf1a and Asf1b," this is quite misleading since not only mammals but also plants and worms have two isoforms of Asf1.

4) (page 6, paragraph 1, lines 11-12) The authors note that "In addition, we only detected minor variations for Asf1a and Asf1b at the RNA level as shown by quantitative RT-PCR during the cell cycle (Supplementary Figure S2C)". However, in Supplementary Figure S2C, mRNA level of Asf1b appear to vary greatly during the cell cycle. The authors should precisely interpret this result.

5) (page 7, paragraph 2, lines 12-13) The authors show that "The rapid upregulation of Asf1b following exit from G0 and entry into the cell cycle is consistent with important cellular demand at early steps prior to S phase". However, there is no data indicating directly that upregulation of Asf1b is required for the exit from G0 and entry into cell cycle. The authors should confirm that reentry into cell cycle does not occur in Asf1b-depleted condition, or change the description.

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7) (page 11, paragraph 3, lines 11-12) The authors note that "Notably, Asf1b proved even stronger than the other proliferation markers p60 and Ki67 (Figure 5A)". However, the correlations with tumor size are quite similar between Asf1b (p=0.0063) and Ki67 (p=0.0066) (Figure 5A). This result should be described more precisely.

8) (page 24, Figure 2C legend, line 4) "See also Supplemental Figure S3B" should be corrected to

"See also Supplemental Figure S4B".

9) (Figure 1B) In the comparison among young, old, and senescent cells of Primary fibroblast IMR90, the expression levels of Asf1b certainly decrease. However, it remains unclear whether the decrease reflects the growth capability of each cell. The correlation between proliferation capacity and expression level of Asf1b should be checked.

10) (Figure 5) The explanations about T0, T1, T2, M0, and N0 should be noted in not only "Materials and Methods" but also "Results" or "Figure legends" to improve readability.

11) (Figure 6A) The results of "Multivariate analysis" should be presented in a table.

12) (Supplementary Figure S3A) While Cyclin A is detected as a single band in Western blot analysis as shown in Figures 1A, 1B and Supplementary Figure S2B, there are two bands of Cyclin A only in Supplementary Figure S3A. The authors should explain the reason.

Referee #2 (Remarks to the Author):

Asf1 is a major histone H3-H4 chaperone involved in both replication-coupled and replication-independent nucleosome assembly pathways. In mammalian cells, there are two Asf1 isoforms, Asf1a and Asf1b. This paper reports that the expression of Asf1b correlates with cell progression, and depletion of Asf1b severely inhibits cell proliferation. Moreover, over-expression of Asf1b correlates with metastasis status of breast cancer, and the level of Asf1b may be used as a new proliferation marker and a prognostic factor of breast cancer.

The demonstration of unique role of Asf1b in cell proliferation and its correlation with the prognosis of breast cancer provide novel insight into the distinct functions of two Asf1 isoforms in humans and would be of general interest to a variety of audience. At the same time, I also had following concerns that need to be addressed before publication of these findings.

1) Depletion of Asf1a and Asf1b has significant effects on transcription of many genes. While deletion of Asf1a and Asf1b affect gene expression distinctly (Figure 3), it is unclear to me whether changes in gene expression contributes to the unique role of Asf1b cell proliferation. Moreover, it is not clear to what extent the differences in gene expression by Asf1a and Asf1b depletion are due to differences in the amounts of Asf1a and Asf1b depleted in cells.

2) It is not clear how depletion of Asf1b results in defect in proliferation. Is this due to activation of DNA damage checkpoint, as authors implied? If this is case, how does deletion of Asf1b, but not Asf1a, results in activation of damage checkpoint?

3) Authors mentioned that Asf1b is a better proliferative marker than p60 and Ki67 in the correlation with clinical parameters such as mitotic cell number and tumor grade (Page 11), and that Asf1b is the only independent prognostic factor for the metastasis free interval (Page 12). Is the sample size bigger enough to allow the authors to make these conclusions? Is it necessary to enlarge the sample volume to compare the prognostic values of those markers, especially in multivariate analysis?

4) Over-expression of Asf1b is correlated with tumor grade and metastasis occurrence. Because depletion of Asf1b affects expression of so many genes, is it possible that the effects of Asf1b in these tumors are also due to their effect of Asf1b on cell migration and invasion? In other word, does depletion of Asf1b U2OS cells or HeLa cells (Figure 4 and Figure 6) also affect cell migration and invasion, in addition to cell proliferation?

Minor point: The word fond changes from places to places in this article (Page 9 and Page 25).

Referee #3 (Remarks to the Author):

The authors have examined Asf1a & b isoforms in human cancer cell lines. The title suggests that Asf1b is needed for proliferation & that it predicts outcome in breast cancer.

The authors used the Hs578Bst and Hs578T model to check levels of the 2 isoforms and to look at the % of cells in different phases of the cell cycle. The find the the Bst cells have lower S phase - 13%, vs the T cells with 25%. The T cells show higher levels of Asf1b RNA vs Asf1a RNA (Panel B), while the protein levels of both look quite similar on the western in panel A.
The Bst cells have equal RNA levels of both but essentially no b protein. Thus they concluse the the b isoform correlates with proliferation - S phase content.
To prove that this is more then a correlation they should have silenced the Asf1b isoform in the T cells & examined the cell cycle profile.

In fig 3 they switch to human U2OS tumor cells - an osteosarcoma model. With these cells they show the silencing of both isoforms does lead to S phase accumulation. In Fig 4, they show that colony formation is more strongly impaired upon loss of Aif1b.

To provide mechanistic insight into breast cancer they should take a panel of breast cancer cell lines, examine Aif1b protein levels & KD the two isoforms independently & together & examine proliferation.

1st Revision - authors' response 28 October 2010

Point by point answer to reviewers

We are thankful to all reviewers for their constructive comments. All suggestions concerning the manuscript and additional experiments were taken into consideration. In this revised version of the manuscript, we have included a number of new experiments and present additional data that specifically address the major concerns, as can be seen with the new set of figures. In addition, we provide additional data for reviewers only which could be included in the figures of the manuscript if needed. *(See "Supplementary data for reviewers" file)*. Altogether, these new results strongly support our conclusion.

For the sake of clarity, we copied each point from the reviewers' comments (in italics) and provide our response below.

Referee #1 (Remarks to the Author):

This manuscript describes the physiological differences between two isoforms of Anti-silencing function 1 (Asf1), Asf1a and Asf1b. The authors report that: I) human Asf1b shows proliferation-dependent expression in cycling and tumoral cells; II) deletion of Asf1b compromises proliferation and leads to aberrant nuclear structure; III) these properties of Asf1b are unparalleled by Asf1a. Further, based on the higher expression level of Asf1b mRNA in breast cancer cells than normal cells, the authors point out that Asf1b could become an effective proliferation marker for breast cancer diagnosis and prognosis. This claim is verified using tissue samples derived from early stage breast tumors. As a whole, the relationships between Asf1b and proliferation, and the effectiveness of Asf1b as a proliferation marker are sufficiently investigated in several types of cells and tissue samples. However, the claim that Asf1b is necessary for proliferation remains debatable. Major and minor
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We have now clarified the Abstract section to make clearer our claims.

2) (page 3) The first paragraph in the "Introduction section" is remotely related to the main claims including the physiological roles of Asf1b in proliferation, and the effectiveness of Asf1b as a proliferation marker in breast cancer. Rewrite of the paragraph or whole constitution of "Introduction" would be of great help for the readers to understand the gist of the manuscript.

We have now reorganized the introduction section with a stronger emphasis on aspects related to breast cancer, proliferation and the need for new prognostic markers.

3) (page 5, paragraph 2) This paragraph is too long. To shorten this paragraph would make the manuscript more readable.

As suggested by the reviewer, we have now shorten this paragraph to improve the manuscript.

4) (Figure 2A) The authors show that tumoral and normal cells contain 25 and 13 % of cells in S phase, respectively (Supplementary Figure S4A). This result suggests the possibility that different expression levels of Asf1s between tumoral and normal cells as shown in Figure 2A do not reflect different properties of tumoral and normal cells, but indicate just the gap of cell cycle between these cell lines. In order to confirm the higher expression level of Asf1b in tumoral cells than normal cells without the effect of different cycling status, the authors should quantify the western blot results in Figure 2A like "Western blot quantification by chemiluminescence" in the "Supplementary Materials and Methods" section.

We now provide quantification of WB in figure R1. We have quantified the amounts of Asf1a and Asf1b proteins in Hs578T and Hs578Bst cells as described in Supplementary Materials and Methods. However, we made the normalisation of Asf1 levels to the Memcode staining since a-tubulin protein also slightly varies between the two cell lines.

This quantification shows that there is about 5.5 fold more Asf1b in the tumoral cell line than in the normal cell line at the protein level. In contrast, Asf1a levels are almost constant (Figure R1).
We agree with the reviewer that there is a difference in the amount of cells in S phase in these two cell lines. However, since Asf1a and Asf1b levels do not vary at the protein level during the cell cycle, making the quantification relative to the amount of cells in S phase does not seem most relevant here. It is rather the number of proliferating cells (as quantified by CAF-1 staining in immunofluorescence) that explains the difference in the levels of Asf1b, which follows the proliferative capacity of the cells.

We have nevertheless performed the quantification as asked by the referee. By taking into account the difference in the cell cycle, the amount of Asf1b is about 4.9 fold higher in the tumoral Hs578T than in the normal Hs578Bst cell line.

5) (Figure 4D) In this manuscript, the claim that Asf1b is necessary for proliferation is only sustained by the colony formation assay as shown in Figure 4D. However, this assay evaluates cell viability, which includes cell death as well as proliferative capacity. Therefore, I'm concerned whether the effect of Asf1b deletion in the assay is attributed only to the perturbation of proliferation. To verify the effects of Asf1b on proliferation, the authors checked the effect of Asf1b deletion on cell cycle (Figure 3A), the localization of a DNA replication-related factor (PCNA) (Supplementary Figure S6C), the amount of γH2A.X as a marker of DNA damage (Supplementary Figure S6D). However, there is no discussion on cell death in Asf1b-deficient cells. The authors should carefully reconsider whether Asf1b is a bona fide regulator necessary for proliferation.

Our aim was to have a global approach to prove the importance of Asf1b for proliferation. We agree with the referee that, in principle, colonies observed by the colony formation assay are a result of effects on proliferation or on cell death or on both. Although we cannot formally exclude a direct effect on cell death, based on its known molecular function as a histone chaperone, it is unlikely that Asf1b is an anti-apopotic factor, which depletion would result in cell death. Rather, we favor the idea that Asf1b is necessary for sustained proliferation. In absence of Asf1b, cells show proliferation defects and this will eventually lead to cell death as a consequence. This explains the reduced number of colonies in Asf1b depleted cells as observed by CFU assay. By looking more closely at our transcriptomic data, we observed a downregulation in the expression of a number of genes important for cell proliferation in Asf1b depleted cells, but not Asf1a, underscoring that cells depleted of Asf1b have a transcriptional signature reflecting troubles in proliferation (Figure R2).
We have now clarified this issue in the main text of the manuscript and modulated our statements.

6) (Figures 3 and 4) One of the main claims of this manuscript is that Asf1b becomes an effective proliferation marker in breast cancer cells. While breast cancer cell lines are used in Figures 1 and 2, an osteosarcoma cell, U-2-OS cell, is used in Figures 3 and 4. To verify the claim in a consistent manner, the authors should use a breast cancer cell, MCF7 cell, for Figures 3 and 4, or explain the reason why the use of U-2-OS cell is appropriate. Even in the latter case, proliferation-dependent expression of Asf1b should be shown in U-2-OS cells as in MCF7 cells (Figure 1A).

We chose the U-2-OS cell line because it is a tumorigenic model cell line with a functional p53 protein widely used in studies implicating cell cycle or checkpoint effects. In addition, the effects of Asf1(a+b) depletion were already extensively characterized in this cell line (Groth et al., Science, 2007) making the comparison easier with our results obtained with single Asf1 depletions. In contrast, the use of MCF-7 cell line seemed appropriate to study the expression of Asf1 proteins in relation to proliferation since these cells can easily and efficiently be arrested into quiescence by serum starvation. In addition, while it is difficult to deplete Asf1 isoforms efficiently in MCF-7 cells, we could obtain a strong downregulation of Asf1 isoforms in U-2-OS cells.

Nevertheless, as requested by the reviewer, we have now performed an arrest of U-2-OS cells into quiescence in order to assess the conservation of the proliferation-dependent expression of Asf1b in these cells. Even with a poor arrest into quiescence (only 10% of CAF-1 negative cells as assessed by immunofluorescence analysis), we could however confirm that Asf1b shows a proliferation-dependent expression by immunofluorescence in single cell analysis thanks to our specific antibodies (Supplementary Figure S3A-B).

Importantly, in addition, we also performed the depletion of Asf1 isoforms in specific breast cancer cell lines in order to strenghten the results obtained concerning the effect of Asf1b on proliferation in the U-2-OS cell line. We now provide as a main figure the results of Asf1a, Asf1b or Asf1(a+b) depletion in the Hs578T breast cancer cell line. Upon depletion of Asf1b alone, but not Asf1a, we observe defects in nuclear morphology, increase in the number of micronuclei and DNA bridges, as well as a dramatic reduction in the number of colonies obtained by CFU assay (Figure 4). These results are consistent with the ones obtained in U-2-OS cells, therefore strenghtening the importance of Asf1b for proliferation. Importantly, these results have also been validated in another breast cancer cell line, MDA-MD-231 (Supplementary Figure S7) and in HeLa cells as well (data not shown, available upon request).

7) (Figure 4D) The authors illustrate the reduced competence of colony formation in the cells treated with siAsf1b or siAsf1(a+b). In the manuscript, the authors described that "Surprisingly, the combined depletion of Asf1(a+b) did not give as strong an effect as Asf1b depletion alone on nuclei morphology and proliferation (Figure 4B and 4D)'(page 10, paragraph 1, lines 14-15). However in Figure 4D, there isn't distinct difference of the survival rates between cells treated with siAsf1b and siAsf1(a+b). The authors should explain how they evaluate the results.

This is a good point raised by the referee. We have now carefully rephrased our results and have removed the word "proliferation" from the sentence, which is now place in the legend of Supplementary Figure 5B. As stated in the manuscript, concerning nuclei morphology, it is not surprising that we do not observe as strong an effect in the Asf1(a+b) depletion compared to Asf1b after 48 hours of depletion since the slow S phase progression observed in this double knockdown

may potentially prevent us from detecting these defects. However, in the CFU assay, we observe the results of a long-term depletion of Asf1 isoforms. While Asf1(a+b) depleted cells eventually go through S phase after 48 hours of depletion, it is therefore possible that the same defects as observed in the Asf1b depletion could occur. The fact that Asf1b and Asf1(a+b) depletions give the same results in CFU assays underscores that Asf1b depletion accounts for most of the effects observed in the double knockdown (see also Figure 3C and Supplementary Figure S4C).

Minor comments:

1) (page 3, paragraph 1, line 9) [Eitoku et al., Cell. Mol. Life Sci., 65, 414-444 (2008)] would also be an appropriate review article since it discusses histone chaperones as a whole.

We thank the reviewer for this useful suggestion and we have now included the reference in the introduction section of the manuscript.

2) (page 3, paragraph 2) The reason why the authors focus on Asf1 should be explained.

This is a good point raised by the reviewer. We have now explained more precisely the choice of the histone chaperone Asf1. Indeed, in various organisms, depletion of the histone chaperone Asf1 leads to S phase progression defects such as in yeast, chicken, drosophila and humans suggesting an essential function of the histone chaperone Asf1 for proliferation.

3) (page 3, paragraph 2, line 6) Although the authors note "mammals possess two Asf1 isoforms, Asf1a and Asf1b," this is quite misleading since not only mammals but also plants and worms have two isoforms of Asf1.

We have now clarified this issue by adding the word "called" in the sentence. There are indeed two isoforms of Asf1 in plants and worms as well as in mammals. However, a careful evolutionary analysis of Asf1 isoforms across eukaryotic evolution (Corpet et al., in preparation) reveals that the distinction between Asf1a and Asf1b is only clear in Amniotes organisms (containing Mammals, Reptiles and Birds). The apparition of two isoforms in plants or worms is an independent event and the two isoforms of Asf1 present in these organisms therefore cannot be called Asf1a and Asf1b.

4) (page 6, paragraph 1, lines 11-12) The authors note that "In addition, we only detected minor variations for Asf1a and Asf1b at the RNA level as shown by quantitative RT-PCR during the cell cycle (Supplementary Figure S2C)". However, in Supplementary Figure S2C, mRNA level of Asf1b appear to vary greatly during the cell cycle. The authors should precisely interpret this result.

We have now described our results more precisely. Eventhough we observed a slight upregulation of Asf1b at the RNA level by quantitative RT-PCR, absence of variation at the protein level suggest that there is no major variation in the quantity of Asf1 isoforms during the cell cycle. The upregulation of Asf1b observed at the RNA level upon S phase entry could be related to the fact that Asf1b can be regulated by E2F1, which is upregulated in S phase (Hayashi et al., DNA and Cell Biology, 2007).

5) (page 7, paragraph 2, lines 12-13) The authors show that "The rapid upregulation of Asf1b following exit from G0 and entry into the cell cycle is consistent with important cellular demand at early steps prior to S phase". However, there is no data indicating directly that upregulation of Asf1b is required for the exit from G0 and entry into cell cycle. The authors should confirm that reentry into cell cycle does not occur in Asf1b-depleted condition, or change the description.

We agree with the reviewer that we have so far no evidence showing that Asf1b is required for the exit from G0. We have therefore removed this sentence.

6) (page 8, paragraph 2, lines 12-13) Although the authors note that "Interestingly, expression of Asf1b paralleled the proliferative status of tumoral cells as assessed by CAF-1 p60 ...", the similar tendency is also observed in normal cells. Therefore, "of tumoral cells" should be erased or changed to "of mammary cells".

We have now made the requested change.

7) (page 11, paragraph 3, lines 11-12) The authors note that "Notably, Asf1b proved even stronger than the other proliferation markers p60 and Ki67 (Figure 5A)". However, the correlations with tumor size are quite similar between Asf1b (p=0.0063) and Ki67 (p=0.0066) (Figure 5A). This result should be described more precisely.

We thank the referee for pointing this inaccurary. We have now carefully stated that "the correlation of Asf1b with the mitotic index and the tumor grade proved even stronger than the other proliferative markers p60 and Ki67 (Figure 5A)".

8) (page 24, Figure 2C legend, line 4) "See also Supplemental Figure S3B" should be corrected to "See also Supplemental Figure S4B".

We apologize for this mistake. Given the large amount of Supplementary Figures, we have now removed this panel which was largely redundant with Figures 2D and 2E.

9) (Figure 1B) In the comparison among young, old, and senescent cells of Primary fibroblast IMR90, the expression levels of Asf1b certainly decrease. However, it remains unclear whether the decrease reflects the growth capability of each cell. The correlation between proliferation capacity and expression level of Asf1b should be checked.

Here, we would like to put forward that the growth capability of each type of cells was studied by Western Blot with CAF-1 p60 staining, which reflects the proliferation capacity of the cells (Polo et al., Cancer Research, 2004). On Figure 1B, we can observe a downregulation of CAF-1 p60 in senescent cells, reflecting a decrease in the proliferation capacity, which is concomitant with Asf1b decrease in expression as well.

10) (Figure 5) The explanations about T0, T1, T2, M0, and N0 should be noted in not only "Materials and Methods" but also "Results" or "Figure legends" to improve readability.

We have now explained in each figure legend (when appropriate) the meaning of T0, T1, T2, M0 and N0.

11) (Figure 6A) The results of "Multivariate analysis" should be presented in a table.

As requested by the reviewer, we now provide a new table with the results of the multivariate analysis performed on 73 patients (breast cancer samples of 1995) (Table 1). In addition, we are now happy to provide an additional table with the results of the multivariate analysis performed on a complete independent set of 62 patients (breast cancer samples of 1996) which validates the analysis performed on the first set of patients (Supplementary Table SIII). We believe that this reinforces the validity of our conclusions putting forward Asf1b as a new prognostic marker of interest in breast cancer.

12) (Supplementary Figure S3A) While Cyclin A is detected as a single band in Western blot analysis as shown in Figures 1A, 1B and Supplementary Figure S2B, there are two bands of Cyclin A only in Supplementary Figure S3A. The authors should explain the reason.

Detection of Cyclin A was performed after CAF-1 p60 in Supplementary Figure S3A, therefore giving us an extra band by Western Blot analysis. We have now stripped the membranes used for detection as following : 2*15 minutes in 25mM Glycine pH=2.0, 2% SDS, followed by two washes in PBS-Tween 0.1% (PBST) and a blocking step in PBST-5% milk for 30 minutes at RT°C. We then reincubated Cyclin A antibody and revealed the antibody as described in Material and Methods. We obtained a single band for the Cyclin A. We have now replaced this new Western Blot in the Supplementary Figure S3C.

Referee #2 (Remarks to the Author):

Asf1 is a major histone H3-H4 chaperone involved in both replication-coupled and replication-independent nucleosome assembly pathways. In mammalian cells, there are two Asf1 isoforms, Asf1a and Asf1b. This paper reports that the expression of Asf1b correlates with cell progression, and depletion of Asf1b severely inhibits cell proliferation. Moreover, over-expression of Asf1b correlates with metastasis status of breast cancer, and the level of Asf1b may be used as a new proliferation marker and a prognostic factor of breast cancer.

The demonstration of unique role of Asf1b in cell proliferation and its correlation with the prognosis of breast cancer provide novel insight into the distinct functions of two Asf1 isoforms in humans and would be of general interest to a variety of audience. At the same time, I also had following concerns that need to be addressed before publication of these findings.

1) Depletion of Asf1a and Asf1b has significant effects on transcription of many genes. While deletion of Asf1a and Asf1b affect gene expression distinctly (Figure 3), it is unclear to me whether changes in gene expression contributes to the unique role of Asf1b cell proliferation. Moreover, it is not clear to what extent the differences in gene expression by Asf1a and Asf1b depletion are due to differences in the amounts of Asf1a and Asf1b depleted in cells.

This is a good point raised by the reviewer. Our depletion analysis is not here to directly assign distinct functions to each Asf1 isoform. Rather the transcriptome analysis underscores for the first time that the two Asf1 isoforms, when depleted, give a very different transcriptional signature. This result was quite surprising given that they share similar molecular functions as histone chaperones. Changes observed were quite subtle, and we thus here consider the transcriptome analysis as an indirect reflection of Asf1b effect on proliferation ('transcriptional signature') rather than the fact that Asf1b directly affects gene related to proliferation.

Concerning the second point, we believe that it it not a slight difference in the levels of Asf1a and Asf1b depletions that would explain such a dramatic difference between the two depletions both on the trancriptome analysis and on the cellular analysis (CFU assays for example). In Supplementary Figure S4, equivalent levels of depletion at the RNA level are obtained between the second set of siRNA against Asf1a (siAsf1a#2) and the first set of siRNA against Asf1b (siAsf1b) (Supplementary Figure S4A). Even when the depletion levels are equivalent, the difference in expression of a subset of genes (eg SRF) is still significant between Asf1a and Asf1b depletions (Supplementary Figure S4B).

In addition, we have now performed the depletion analysis in the breast cancer cell line Hs578T in which we could obtained a better depletion of Asf1a than Asf1b (See new Supplementary Figure S6C). Even in this case, we can still observe a dramatic difference in the number of colonies as visualized by CFU assay after depletion of one or the other Asf1 isoform (New Figure 4D). This therefore strenghtens the fact that a specific function in proliferation can be assigned to Asf1b.

2) It is not clear how depletion of Asf1b results in defect in proliferation. Is this due to activation of DNA damage checkpoint, as authors implied? If this is case, how does deletion of Asf1b, but not Asf1a, results in activation of damage checkpoint?

We do not intend to claim that Asf1b depletion results in DNA damage checkpoint activation which would lead to proliferation defects. Checkpoint activation was extensively studied upon Asf1(a+b) depletion in Groth et al., Science, 2007, and this showed that there is no checkpoint activation upon Asf1 isoforms knowdown.

Rather, DNA damage apparition (eg gH2A.X) is a late consequence of Asf1b depletion (See Supplementary Figure S9D), possibly reflecting accumulating defects in chromatin assembly or even cell death. Thus, we believe that occurence of DNA damage is a consequence of Asf1b impact on proliferation rather than a cause.

In the discussion of the manuscript, we put forward two hypotheses to explain the specific role of Asf1b on proliferation. First, Asf1b could be the major isoform acting during replication as a histone donor/acceptor. This is possible given the fact that (i) Asf1b contributes to a major extent to the

defects observed upon Asf1(a+b) knockdown (in particular slow S phase progression); (ii) the transcriptional signature observed upon Asf1b depletion links this isoform with replication (Figure 3D), and (iii) we also observed a reproducible and slight increase in the proportion of cell in S phase in HeLa and Hs578T cells depleted of Asf1b (Figure R4) underscoring that without Asf1b alone, cells have troubles going through S phase. The second hypothesis is that Asf1b would have a specific function outside S phase that is required for proliferation. In particular, it is possible that Asf1b could play a specific role during mitosis given the increase in the number of micronuclei, the altered nuclei and the presence of DNA bridges that we observe upon depletion of this isoform in various cell lines (See new Figure 4 and new Supplementary Figure 7 in addition to Supplementary Figure S5). These two hypothesis are not mutually exclusive and would require an important amount of work to be tested in depth.

3) Authors mentioned that Asf1b is a better proliferative marker than p60 and Ki67 in the correlation with clinical parameters such as mitotic cell number and tumor grade (Page 11), and that Asf1b is the only independent prognostic factor for the metastasis free interval (Page 12). Is the sample size bigger enough to allow the authors to make these conclusions? Is it necessary to enlarge the sample volume to compare the prognostic values of those markers, especially in multivariate analysis?

We agree with this reviewer that the sample size was quite limited. We have now repeated the QPCR analysis on an independent new set of 71 small breast tumors extracted in 1996 in Institut Curie. This new dataset extends and confirms the results obtained with the previous set (See new Supplementary Figures 9B-C and Supplementary Table SIII).
In particular, in univariate analysis, we confirmed that high levels of Asf1b are associated with increased rates of disease progression and metastasis occurrence in small breast cancer (Supplementary Figures 9C). In addition, in multivariate analysis, we confirmed the high prognostic value of Asf1b for predicting metastasis occurence, over known markers such as CAF-1 p60 and Ki67. Interestingly, in this second set of data, we also find Asf1b as a marker of prognostic value for predicting the disease recurrence (DFI) in multivariate analysis (Supplementary Table SIII).

Of note however, we could not pool the two sets of data together to perform the multivariate analysis since the QPCRs were performed with different QPCR amplification kits (ABI versus KAPA Biosystems). Since values obtained for the genes could vary depending on the QPCR kit, we could not directly combine the data from the two sets. Instead, we believe that the validation of the prognostic value of Asf1b on an independent set of 71 breast tumor samples, strongly reinforces our conclusions putting forward Asf1b as a new prognostic marker of interest and as an attractive target for breast cancer treatment.

4) Over-expression of Asf1b is correlated with tumor grade and metastasis occurrence. Because depletion of Asf1b affects expression of so many genes, is it possible that the effects of Asf1b in these tumors are also due to their effect of Asf1b on cell migration and invasion? In other word, does depletion of Asf1b U2OS cells or HeLa cells (Figure 4 and Figure 6) also affect cell migration and invasion, in addition to cell proliferation?

We thank the reviewer for this useful suggestion. Indeed, while depletion of Asf1b affects expression of many genes, we cannot exclude that this could have an impact on cell migration and invasion. We checked whether know genes implicated in metastasis were specifically affected upon Asf1b depletion but could not find any obvious evidence for this (Figure R2). Indeed, we found that expression of the matrix metalloproteinase (MMP) MMP1, which is involved in breast cancer metastasis to lung (Minn et al., Nature, 2005) is specifically increased, while the TIMP3 inhibitor of MMP, which is a metastasis suppressor, is downregulated in Asf1b depleted cells. However we could also found an increase of MMP9 (Figure R2) or MMP3 (not shown) both in Asf1a and Asf1b depleted cells (Figure R2), thus underlining that the situation is not simple. In addition, we did not find any variations in the E-cadherin or the avb3 integrin, which are other known positive regulators of metastasis (Sloan and Anderson, CMLS review, 2002). Nevertheless, it is interesting to note that MDA-MB-231 cells, which are highly metastatic, have a 25 fold increase in Asf1b expression compared to the normal mammary HMEC cell line, while Asf1a levels are constant (Figure R4). We therefore believe that investigating the specific role of Asf1b in metastasis occurrence could constitute the object of a whole separate study and is currently beyond the scope of this manuscript.

Minor point: The word fond changes from places to places in this article (Page 9 and Page 25).

We thank the reviewer for noticing this change which we have now corrected.

Referee #3 (Remarks to the Author):

The authors have examined Asf1a & b isoforms in human cancer cell lines. The title suggests that Asf1b is needed for proliferation & that it predicts outcome in breast cancer.

The authors used the Hs578Bst and Hs578T model to check levels of the 2 isoforms and to look at the % of cells in different phases of the cell cycle. The find the the Bst cells have lower S phase - 13%, vs the T cells with 25%. The T cells show higher levels of Asf1b RNA vs Asf1a RNA (Panel B), while the protein levels of both look quite similar on the western in panel A.
The Bst cells have equal RNA levels of both but essentially no b protein. Thus they concluse the the b isoform correlates with proliferation - S phase content.

We would like to point out that results from RT-QPCR were set to 100% in Hs578Bst cells in order to visualise the increase in Asf1b expression in tumoral cells. However, of course, this is only an arbitrary representation which does not reflect that Asf1a and Asf1b protein levels in tumoral cells are quite similar. In addition, we do not claim that Asf1b correlates with S phase content, but only with proliferation in general (see below).

To prove that this is more than a correlation they should have silenced the Asf1b isoform in the T cells & examined the cell cycle profile.

We have now performed Asf1a/b/(a+b) depletion in Hs578T cells and examined both cell cycle profile, as well as CFU capacity and cellular morphology (New Figure 4 and Supplementary Figure S6). As oserved in U-2-OS cells, the depletion of Asf1b alone did not dramatically altered the cell cycle profile althought a slight increase in the number of cells in S/G2 could be observed (Figure R3). We consistently observed an important increase in the number of cells in S phase upon knockdown of both Asf1 isoforms, as observed in the U-2-0S cell line (this study and Groth et al., Science, 2007). Importantly, these observations were also confirmed in another breast cancer cell line, MDA-MB-231 (New Supplementary Figure S7).

One hypothesis that we put forward in the discussion is that Asf1b would be the major isoform acting as a histone donor/acceptor during replication, and this could explain its critical requirement for proliferation. This is possible given the fact that (i) Asf1b contributes to a major extent to the defects observed upon Asf1(a+b) knockdown (in particular slow S phase progression); (ii) the transcriptional signature observed upon Asf1b depletion links this isoform with replication (Figure 3D), and (iii) we also observed a reproducible and slight increase in the proportion of cell in S phase in HeLa and Hs578T cells depleted of Asf1b (Figure R4) underscoring that without Asf1b alone, cells have difficulties going through S phase.

In fig 3 they switch to human U2OS tumor cells - an osteosarcoma model. With these cells they show the silencing of both isoforms does lead to S phase accumulation. In Fig 4, they show that colony formation is more strongly impaired upon loss of Aif1b.
To provide mechanistic insight into breast cancer they should take a panel of breast cancer cell lines, examine Aif1b protein levels & KD the two isoforms independently & together & examine proliferation.

We have now examined Asf1a and Asf1b levels in a panel of breast cancer cell lines by quantitative RT-PCR. We show that Asf1a and Asf1b levels are comparable in normal breast cell lines such as HMEC cells compared to our reference gene RPLPO. However, Asf1b is highly overexpressed in all breast cancer cell lines (MDA-MB-231, SKBR3, ZR75.1) which are highly proliferative, while Asf1a is only slightly overexpressed (Figure R4). This strenghtens and extends our previous results that Asf1b expression levels correlates with proliferation.

In addition, we have also performed the depletion of Asf1 isoforms in specific breast cancer cell lines in order to strenghten the results obtained concerning the effect of Asf1b on proliferation in the U-2-OS cell line. We now provide as a main figure the results of Asf1a, Asf1b or Asf1(a+b) depletion in the Hs578T breast cancer cell line (New Figure 4 and Supplementary Figure S6) and in the supplementary figures the MDA-MB-231 cell line (Supplementary Figure S7). Upon depletion of Asf1b alone, but not Asf1a, we observe defects in nuclear morphology, increase in the number of micronuclei and DNA bridges, as well as a dramatic reduction in the number of colonies obtained by CFU assay (Figure 4 and Supplementary Figure S7). With these results, which are entirely consistent with the ones obtained in U-2-OS cells, we have therefore reinforced the importance of Asf1b isoform, but not Asf1a, for proliferation.

2nd Editorial Decision 15 November 2010

I have received comments from the two referees I asked to review the revised version of your Asf1b manuscript. As you will see below they both support publication in The EMBO Journal pending some minor revisions. I would be grateful if you could comment of point #2 and also if it is quick and possible to add a sentence to the manuscript addressing point #3. Since your earlier response to the authors reports contains some figures for the referees, I would like to ask if you are happy that theses are included in the final Review Process File that contains the referee reports and additional correspondence that will be published alongside the final paper. Finally, since it should be straightforward to include these changes to the manuscript, it may be more efficient just to send the Word file by email that can be directly replaced and therefore the figures do not need to be uploaded once more.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website:
http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to reading the revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The revised manuscript by Corpet et al. has been improved significantly by the added data that strengthen the authors' claims. In particular, the result that breast cancer cell line, Hs578T cells, exhibits the same phenotypes as those obtained by using osteosarcome cell line, U-2-OS cells (Figure 4), clearly supports the authors' argument. The reconstruction of Abstract and Introduction sections contributes to the improvement of the manuscript, too. It is well written overall. However, there are a couple of points to consider for further improvement before being accepted for publication.

1) (page 5, last paragraph) The last paragraph in Introduction section still can be shortened. There would be no need to detail the results in Introduction section. If the authors would like to refer to the results there, more concise description would be preferable.

2) (Figure 2B and Supplementary Figure S2) In Figure 2B, the authors claimed that the protein level of Asf1b is higher in Hs578T tumor cells than in Hs578Bst normal cells. The addition of %CAF-1 as a proliferative marker is supportive to their claim. They also explained that the difference of the Asf1b level between tumor and normal cells is independent of the differences of cell cycle between these cells, based on the results of Figure S2C and the previous paper (Sillje and Nigg, Curr. Biol. 2001). In Figure S2C, however, the amounts of loaded total cell extracts (1x and 2x) do not appear to be dose-dependent. I'm afraid that this data may have low accuracy as compared to other data and negatively affect the reliability of the manuscript.

3) (page 10, first paragraph) The authors mentioned about the results of Figure 4D that "given the decrease in a number of genes required for proliferation in Asf1b depleted cells, but not Asf1a, in out transcriptomic data (data not shown), the effects observed upon Asf1b depletion most likely reflect an acute effect on proliferation leading to cell death as a consequence, rather than a direct effect on cell death." and they claimed that the decrease of colony number of Asf1b-depleted cells would be directly caused by the defect of proliferation, rather than cell death (also see Figure R2 in the point-by-point answers for the previous review). To further support the notion, the authors might want to examine the effects of Asf1b depletion on the genes involved in cell death, besides on those involved in proliferation, using the transcriptomic data like Figure R2.

Referee #2 (Remarks to the Author):

The revised manuscript has addressed my concerns and I now support its publication in EMBO J.

2nd Revision - authors' response 19 November 2010

We are thankful to all reviewers for considering our revised manuscript and for supporting publication in The EMBO Journal. We have now included all required comments in the main text of the manuscript.
For the sake of clarity, we copied each point from the reviewers' comments (in italics) and provide our response below.

Referee #1 (Remarks to the Author):

The revised manuscript by Corpet et al. has been improved significantly by the added data that strengthen the authors' claims. In particular, the result that breast cancer cell line, Hs578T cells, exhibits the same phenotypes as those obtained by using osteosarcome cell line, U-2-OS cells (Figure 4), clearly supports the authors' argument. The reconstruction of Abstract and Introduction sections contributes to the improvement of the manuscript, too. It is well written overall. However, there are a couple of points to consider for further improvement before being accepted for publication.

We thank the reviewer for his constructive comments.

1) (page 5, last paragraph) The last paragraph in Introduction section still can be shortened. There would be no need to detail the results in Introduction section. If the authors would like to refer to the results there, more concise description would be preferable.

We have now extensively shorten this paragraph to only keep the essential message without going into the details of the results.

2) (Figure 2B and Supplementary Figure S2) In Figure 2B, the authors claimed that the protein level of Asf1b is higher in Hs578T tumor cells than in Hs578Bst normal cells. The addition of %CAF-1 as a proliferative marker is supportive to their claim. They also explained that the difference of the Asf1b level between tumor and normal cells is independent of the differences of cell cycle between these cells, based on the results of Figure S2C and the previous paper (Sillje and Nigg, Curr. Biol. 2001). In Figure S2C, however, the amounts of loaded total cell extracts (1x and 2x) do not appear to be dose-dependent. I'm afraid that this data may have low accuracy as compared to other data and negatively affect the reliability of the manuscript.

We understand the concern of the reviewer about Figure S2C and thus reexamined carefully the data to convince ourselves. The loading control (Memcode staining) together with CAF-1 p60 staining show that there is an increase in the amount of loaded total cell extracts between 1X and 2X samples. In order to clarify this, we have done two things : (1) we have now put the Memcode staining in a gray scale which should make it easier to see the loading differences and (2) we have now quantify the loading based on the Memcode staining and provide a curve of the intensity of the bands (arbitrary units) (new Figure S2C). This clearly shows that there is a dose-dependant increase in the intensity of the bands. We believe that even if the loading is not perfectly equal between time samples, this shows that there is no major variation in Asf1a/b amounts during cell cycle, in contrast to the variation observed for Cyclin A.

3) (page 10, first paragraph) The authors mentioned about the results of Figure 4D that "given the decrease in a number of genes required for proliferation in Asf1b depleted cells, but not Asf1a, in out transcriptomic data (data not shown), the effects observed upon Asf1b depletion most likely reflect an acute effect on proliferation leading to cell death as a consequence, rather than a direct effect on cell death." and they claimed that the decrease of colony number of Asf1b-depleted cells would be directly caused by the defect of proliferation, rather than cell death (also see Figure R2 in the point-by-point answers for the previous review). To further support the notion, the authors might want to examine the effects of Asf1b depletion on the genes involved in cell death, besides on those involved in proliferation, using the transcriptomic data like Figure R2.

We thank the reviewer for underlining this point. We have now looked extensively in our transcriptomic data and could not find any bias towards an activation of pro-apoptotic genes or a downregulation of anti-apopotic genes specifically in Asf1b depleted cells (new Figure R5). We have now carefully discussed this point in the manuscript as requested by the reviewer.

Referee #2 (Remarks to the Author):

The revised manuscript has addressed my concerns and I now support its publication in EMBO J.

We thank the reviewer for being positive about our revised version of the manuscript.

3rd Editorial Decision 23 November 2010

I have looked through the revised version of your manuscript and I find that you have addressed all the remaining issues. I am happy to accept the manuscript for publication in The EMBO Journal. You will receive the official acceptance letter in the next day or so.

Yours sincerely,

Editor
The EMBO Journal

