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# The *hSSB1* orthologue *Obfc2b* is essential for skeletogenesis but dispensable for the DNA damage response *in vivo*

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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 28 June 2012

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received the comments of three expert reviewers, which you will find copied below. I am pleased to inform you that all referees consider your findings of importance and interest and are therefore in favor of eventual publication in The EMBO Journal. Nevertheless there are a number of concerns that will need to be addressed prior to acceptance. In particular, referees 1 and 3 raise several specific points related to the assessment of DNA damage response phenotypes and their basis which I feel will be important to address.

On the other hand, referee 2 also has various issues with the analyses and descriptions of the skeletal and developmental phenotypes, which in my opinion may be less critical for the main conclusions and message of the current manuscript, and whose complete addressing might also be beyond the scope of a regular revision of this work - I would therefore not insist on these experiments for eventual acceptance of a revised version of the manuscript. Nevertheless some limited further characterization of the developmental aspects should be conducted simply to directly support the interpretation that observed developmental defects are in fact only related to apoptosis following replicative stress; e.g. by confirming that selected bone/cartilage differentiation markers are not affected in the KO mice. Furthermore, I feel that following the suggestion in referee 3's point 3 would also be important to help clarify this issue. Finally, I think these aspects of the manuscript would be greatly strengthened by referencing and discussing the precedent of skeletal defects observed also in other genomic instability syndromes such as Seckel Syndrome.

 

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, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you again for the opportunity to consider your work for publication - I look forward to your revision. Please do not hesitate to contact me should you have any further questions regarding this decision or the revision requirements!

Yours sincerely,

Editor
The EMBO Journal

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REFEREE COMMENTS

Referee #1

In this manuscript, Feldhahn et al. study the role of single-stranded DNA-binding protein 1 (SSB1) by generating knockout mice for Obfc2b, the orthologue to the human SSB1 gene. They find that loss of Obfc2b results in perinatal lethality characterized by abnormal skeletal development and growth delay. This phenotype is associated with increased skeletal apoptosis, which can be partly rescued by p53 deletion. Unexpectedly, and in contrast to previous reports studying the function of human SSB1 by knockdown experiments, the authors find no evidence that Obfc2b is essential for the DNA damage response: targeted loss of Obfc2b specifically in B lymphocytes had no effect on B cell development, which requires V(D)J recombination, or on class switch recombination. Also T cell development did not require Obfc2b. Furthermore, Obfc2b-deficient cells were not hypersensitive to ionizing radiation and showed normal DNA damage checkpoint activation. Together, these results suggest that Obfc2b is essential for skeletogenesis and viability, but not for the DNA damage response. Of note, Obfc2b deficiency induced compensatory up-regulation of Obfc2a (orthologue to SSB2) but this did not seem to affect the DNA damage response to irradiation either. Instead, Obfc2b (SSB1) and Obfc2a (SSB2) seem to have redundant functions for genome stability in the context of proliferation.

This is a timely and topical study. The first report in 2008 on the SSB1 involvement in genome stability sparked a lot of attention but the exact function of SSB1/2 remains elusive due to the perplexing and ever-growing number of DNA damage-associated events these proteins seem to regulate. This new work, using a clean genetic knockout model, is an important contribution to this debate and has the potential to give new impulses towards a better understanding of the function of these single-stranded DNA-binding proteins. The findings are unexpected, the conclusions provocative, and as such this study should be seen by the field. However, the current data also raises several questions, which should be clarified before publication. Specifically, two main issues need to be addressed: Proper assessment of DNA damage response on time and dose scales, and more insight into how Obfc2a (SSB2) deficiency causes spontaneous DNA damage. The following points summarize suggestions and ideas of how this can be achieved.

1) The conclusion that Obfc2b does not seem to be required for the DNA damage response to IR will be surprising to many. However, most of the data in this manuscript have been derived from a single dose of IR or a single time-point, and the conclusion would be much stronger if corroborated. First, it would be informative to test proper G2/M checkpoint activation (Figure 5A) also at lower doses of IR from 0.25 Gy to 2 Gy, where checkpoint defects can be observed more easily. Second,

 

the G1/S checkpoint assay (Figure 5B) should be complemented with additional time-points, e.g. 1h, 2h, 4h and 12h and the combined results could be displayed in a chart in addition to the histograms for better readability. Third, the panel on IR-induced phosphorylations (Figure 5C) should be extended by including different recovery times after IR, ranging from for example 5 minutes (early signaling) to 4 hours (sustained signaling) rather than showing a single time-point only. Finally, can the authors assess cellular survival / proliferation in response to IR of Obfc2b-deficient cells? Similar criteria apply also for the key experiments with the Obfc2b/Obfc2a double deficient cells.

2) On a more general note (linked to the previous point): As the authors outline already in the introduction, their findings are in conflict with proposed functions of SSB1 and SSB2 based on experiments in human cell lines by different labs. The authors should thus include a dedicated paragraph in the discussion to address what, in their opinion, can cause such differences and whether and how they could be reconciled.

3) The authors suggest a (potentially redundant) function of Obfc2b/Obfc2a for cellular proliferation, and Obfc2a depletion results in reduced levels of p-Chk1 after IR, spontaneous accumulation of phosphorylated H2ax and in increased genomic aberrations. These observations are very interesting and could indicate that Obfc2b/Obfc2a are important to prevent replicative stress, possibly in a similar manner as the ssDNA binding protein Rpa1. Could the authors extend their analyses of this phenotype and test whether Obfc2b-, Obfc2a-, and Obfc2b/Obfc2a-deficient cells are hypersensitive to experimentally induced replication stress, e.g. by addition of hydroxyurea or aphidicoline? Are ATR-dependent signaling and phosphorylation of downstream targets or Rpa1-loading in response to replicative stress affected? Is cellular survival in dose-response to hydroxyurea affected? Experiments in these directions could help better define this phenotype and even shed light on redundant versus independent functions of Obfc2b and Obfc2a.

4) A technical remark to the previous point: The observation that Obfc2a knockdown by shRNA causes spontaneous DNA damage (Fig. 7B) is intriguing but more controls should be provided to exclude that this increase in DDR signaling is not an unwanted byproduct of the shRNA treatment. For instance, the authors may knockdown Obfc2b and show that this does not increase γH2ax (consistently to what se see after genetic knockout).

5) Fig. 5B should be supplemented with an appropriate control (ATM inhibitor, ATM -/- cells) to show that the G1/S checkpoint defect can be detected under these conditions.

Referee #2

Feldhahn and collaborators report a loss-of-function study of the role of Obfc2b (mouse orthologue of hSSB1) in mice. It has been previously published that hSSB1 regulates the DNA damage response in cell lines in vitro. Here, the authors present in vivo and in vitro data, which collectively argue convincingly against such role, in mouse embryonic fibroblasts, and in B lymphocytes. On the other hand, they present data demonstrating that Obfc2b plays a critical role in skeletal development. The authors suggest that most of the skeletal abnormalities observed in Obfc2b deficient mice result from increased cell apoptosis.

The work of Feldhahn and colleagues is overall of good quality. Their findings are both novel and important since they describe a new and unexpected role of Obfc2 in skeletal development, and since their data invite the scientific community to reconsider the accepted idea that all single-stranded DNA-binding proteins are necessarily critical for the DNA damage response. However, there are several points (detailed below) to address, in order to improve the quality of this manuscript. In particular, the skeletal phenotype has not been analyzed thoroughly enough. Moreover, the manuscript appears poorly written.

Main point #1: Obfc2b expression pattern could be better described, in particular in tissues from which skeletal elements originate. Obfc2b expression should be analyzed at various developmental

 

stages in addition to E11.5 (earlier and later stages). It is important to present sections of limb buds (Forelimb and hindlimb at different stages), to show clearly whether this gene is expressed in the limb ectoderm and/or the limb mesoderm. This expression could be compared with that of specific markers of the limb ectoderm/mesoderm. Any differences between forelimb and hindlimb buds should be stated and discussed. It is also important to perform in situ hybridization on sections of developing bones at later stages of skeletal development (for example, E14.5, E16.6, and E18.5) to show where Obfc2b is expressed in the developing growth plate cartilage, and in the bone compartment (in tibias for example). Classical cartilage markers (Col2, Ihh, ColX, etc.) and bone markers (Col1, OPN, etc.) should be utilized on adjacent sections to mark the different cell types (chondrocytes versus osteoblasts), and the different layers of the growth plate reflecting various degrees of maturation of chondrocytes.

Main point#2: The distribution of classical cartilage and bone markers (mentioned above) should be analyzed in Obfc2b deficient mice, on sections of limbs harvested at different stages of endochondral bone development (E14.4, E16.5, E18.5 for example). Better images illustrating the cleft palate defect could be provided (many examples of what people present can be found in published articles). Cell proliferation (phospho-histone H3, for example), and cell death should be evaluated on sections of mutant and control limbs at various stages of skeletal development (E14.4, E16.5, E18.5). Quantifications of any relevant differences observed should be presented (with error bars and p values). For all tissue samples, an adjacent section should be stained with H&E to show clearly the histology of control and mutant skeletal elements. TUNEL analyses should also be performed at stages earlier than E12.5 presented in Fig. 3A (E9.5, and/or E10.5 for example). Indeed, it is important to evaluate cell death in the limb ectoderm and limb mesoderm prior to chondrocyte differentiation. These observations could explain at least in part the missing digits and should be discussed accordingly. In fact, it could be interesting to analyze the effect of the loss of Cbfc2b on the expression of Shh and FGFs in the limb ectoderm since these molecules control autopod patterning, which is particularly affected in Cbfc2b deficient mice. TUNEL analyses should also be done in Cbf2b;p53 double KO mice to show that cell death is "rescued" in at least some skeletal elements of double mutant mice. The authors should not hesitate to blow up their pictures to better illustrate the cell death in the developing limb and elsewhere. Dapi images presented in figure 3A should be replaced by H&E staining. High magnification DAPI images together of high magnification TUNEL images (to show groups of cells rather than a large view of a tissue) should still be presented.

Additional comments to improve the quality of this manuscript:

- The discrepancy between what has been published about the role of hSSB1 in the DNA damage response, and the authors' data could be further discussed. The authors point out the fact that previous studies have been done in vitro, while they present in vivo data arguing against the conclusions of this former work. One can understand that results obtained in vivo may differ from what has been obtained in vitro, which may be less physiologically relevant. However, the authors also present several in vitro data, which differ from other in vitro results published previously. Is there any possibility to reconcile the old published data with their new data? It seems important to provide more elements of reflection to the readers, to understand why this works leads to different conclusions.

- The authors could also discuss further why Obfc2b deficiency leads essentially to skeletal defects, while other tissues seem relatively intact. The compensatory increase of Obfc2a seems to occur in several tissues, including in ribs (Fig.6A), yet Obfc2a does not compensate for the loss of Obfc2b in this tissue. Is the ratio of Obfc2b/Obfc2a expression (physiological expression) different in skeletal tissues compared to other tissues? Would other genes compensate for the loss of Obfc2b in other tissues?

- The manuscript is overall not written well enough. This is particularly true for the abstract. For example, the authors write that Obfc2a and Obfc2b may have overlapping functions, and then write: "Consistent with this idea, we show that Obfc2b is not required for the initiation of DNA damage checkpoints and for maintenance of genomic stability in B lymphocytes and primary fibroblasts". It is hard to understand why it is consistent, not knowing what Obcfc2a function is. This sentence would actually suggest that Obcfc2a like Obcf2b is not required for the initiation of the DNA damage checkpoint and maintenance of genomic stability (if their functions are similar). What the

 

authors mean, most likely, is that Obfc2a may play redundant functions with Obfc2b, and thus compensate for the loss of Obfc2b in deficient mice. This sentence makes the abstract confusing, especially since it opposes abruptly the first information mentioned in the abstract (hSSB1 is essential for the initiation of DNA damage checkpoint), without any explanation of this major discrepancy. The abstract should perhaps emphasize the physiological function of Cbfc2b, rather than the redundancy with Cbfc2a, which is too "central" in the current abstract.

- The titles of each paragraph in the result section are inappropriate, and/or inaccurate. For example, "increased skeletal apoptosis" sounds awkward because it is not clear what "skeletal apoptosis" exactly is. It is also not really accurate since apoptosis detected in the limb buds seems restricted to the limb ectoderm (in non skeletal tissue; see fig 3A). The title "Obfc2b is redundant in B lymphocytes and MEFs" is not particularly attractive since it does not say with what Obfc2c is redundant with. It is also not accurate since the content of this paragraph does not demonstrate that Obfc2b is redundant with anything, but rather dispensable for specific functions. This title also does not say anything about the role of this gene in the DNA damage response, which is the main point of this paragraph. Several additional titles are not particularly attractive, and could be modify to formulate precisely the conclusion of the data presented.

- The manuscript is somewhat hard to read for people who are not familiar with the DNA damage response. Several acronyms are not spelled out, and the functions of several molecules are not described throughout the manuscript (gammaH2ax, ATM, MRN, CTIP, Ku80, Xrcc4, Lig4, etc.). Mentioning that gammaH2ax accumulates is meaningless to someone who does not know what it is.

- The rational for doing several experiments is not clearly presented in the manuscript.

- Control (wild-type, or double heterozygous) littermates should be shown in figure 3B, where the skeletal phenotype of Obfc2b and p53 double deficient mice is presented.

Referee #3

In the present work, Feldhahn and colleagues describe the phenotype of mice deficient in SSB1. Knockout mice die in utero with skeletal problems, which can be only partially rescued by p53-deficiency. SSB1 deficient cells overexpress SSB2, which could be compensating the loss of SSB2 (or not...). Since the authors have a conditional allele, they then generated SSB1-deficient B cells. This allowed them to perform a comprehensive analysis of the role of SSB1 in genome maintenance. Surprisingly, the authors find that SSB1 is NOT needed for checkpoint activation nor for overall DNA repair activities in B cells. The role of SSB1 seems to be restrained to skeletal tissue, which suffers from apoptosis during embryonic development. In contrast to SSB1, shRNA mediated depletion of SSB2 in MEF is sufficient to lead to genomic instability, suggesting a more general role for this factor. Nevertheless, SSB2 depletion does also NOT impact on ATM-activation, which sharply contrasts with the previous literature.

In 1998, Kum Kum Khanna and colleagues (Richard DJ et al Nature 1998) reported that SSB1 is a master regulator of (almost all...) DNA repair and checkpoint activities in a mammalian cell. It was essential for ATM activation, NHEJ, HR, ... On a personal note, the extreme pleotropic effects of SSB1 described in this original MS already made me wonder about the strength of some of the conclusions of the work. In fact, with a couple of exceptions, not much follow-up has been made on this supposed broad-regulator of the DDR.

To my eyes, the work presented here clarifies this issue and -although a negative result- it will be of great interest to the readers of EMBO Journal.

The data are of superb quality and I believe sufficient to make the point that the authors are trying to make. Having said that I have a couple of minor comments and suggestions, which I believe, could make the manuscript stronger.

1. Text: SSB1 deficiency is not essential for ATM activation. This is an important message (since it

 

contrasts the previous works; i.e. from KK Khanna or M Pagano) and should be emphasized in the abstract.

2. Text: Skeletal (including skull) problems are also seeing in other genomic instability syndromes, including ATR mutations (Seckel Syndrome) that compromise the response to ssDNA. The authors could use this information for their discussion.

3. Experiment: The authors see TUNEL on the developing bone but, do they see DNA breaks and or an accumulation of single stranded DNA? It would be nice to look for H2AX-P and 53BP1 foci (for breaks), or RPA foci (for ssDNA). It would help to understand whether the skeletal defects are also related to genome maintenance, or a completely independent role of SSB1 in bone development.

4. Experiment: If one looks at the data presented in Fig. S3, it looks to me like SSB2 depleted cells have normal levels of p53 phosphorylation, but reduced levels of Chk1 phosphorylation. If this is true, this is an important observation since it would suggest that SSB2 is important for ATR and not ATM activation. This is similar then to the role RPA -another ssDNA binding protein- in ATR activation. To clarify whether this is the case, I would suggest that the authors: (1) Look at Chk1 phosphorylation in response to hydroxyurea (which is a much better activator of ATR than IR) and (b) verify that replication rates are not lower on SSB2 depleted cells. If replication levels are lower, then it would be inconclusive to see lower levels of HU-induced Chk1-P (since this event occurs during replication).

1st Revision - authors' response 01 August 2012

Thank you for the rapid handling of our manuscript and the suggestions for how to improve it. The reviews were positive and constructive. In response we performed additional experiments, which are included in a revised manuscript.

Regarding the concerns that you emphasized we have done the following:

- We repeated our experiments on irradiated B cells including additional doses, recovery times and positive controls as requested by referee #1 (please see Figure 5 and Supplemental Figure 6C).
- To address the question of whether the developmental defect is only related to apoptosis in early development or if the function of bone forming cells is also affected (asked by referee #2) we analyzed primary chondrocytes, osteoblasts and osteoclasts from *Obfc2b*$^{-/-}$ vs. wildtype mice by gene array (please see Supplemental Figure 3). We find that besides *Obfc2b* no other gene is significantly deregulated in *Obfc2b*$^{-/-}$ cells. Thereby showing that at later stages of development chondrocyte, osteoblast and osteoclast function is not affected by *Obfc2b* loss. In agreement with this, we additionally show that *Obfc2b*$^{-/-}$ embryos exhibit a defect in mesenchymal condensation (see Figure 3D) that is required for bone formation and occurs before the development of chondrocytes, osteoblasts and osteoclasts. These effects occur at the time point when we also see apoptosis (E12.5) in *Obfc2b*$^{-/-}$ embryos.
- In order to determine if apoptosis in *Obfc2b*$^{-/-}$ embryos is related to increased genomic damage we performed Western blotting on fore- and hindlimbs of *Obfc2b*$^{-/-}$ and wildtype embryos from E12.5. This is the time point when we also see apoptosis in *Obfc2b*$^{-/-}$ embryos. We find increased gH2ax accumulation and p53 phosphorylation at serine 15 in *Obfc2b*$^{-/-}$ embryos, both of which are induced by and serve as a marker for DNA damage (please see Figure 3C).
- As suggested by you and the referees we included a new paragraph in the discussion addressing genomic instability associated developmental disorders including the Seckel Syndrome and a dedicated paragraph addressing the discrepancy between our findings and previously published data.

In summary, the revised manuscript provides additional evidence to support the idea that tissue specific expression of *Obfc2b* helps to prevent apoptosis due to replication-associated DNA damage in skeletal compartments. By doing so *Obfc2b* ensures the correct development of skeletal structures during early embryogenesis (please see Figure 8).

 

Please find a detailed response to the referee queries below.

Reply to the referees:

***Referee #1***

*1) The conclusion that Obfc2b does not seem to be required for the DNA damage response to IR will be surprising to many. However, most of the data in this manuscript have been derived from a single dose of IR or a single time-point, and the conclusion would be much stronger if corroborated. First, it would be informative to test proper G2/M checkpoint activation (Figure 5A) also at lower doses of IR from 0.25 Gy to 2 Gy, where checkpoint defects can be observed more easily.*

This is an excellent suggestion. The revised Figure 5A includes the requested lower doses of irradiation for the analysis of the G2/M checkpoint. The data confirms our initial observation that loss of *Obfc2b* does not affect the initiation of the G2/M checkpoint while inhibition of ATM interferes with G2/M checkpoint activation.

*Second, the G1/S checkpoint assay (Figure 5B) should be complemented with additional time-points, e.g. 1h, 2h, 4h and 12h and the combined results could be displayed in a chart in addition to the histograms for better readability.*

Good point. We repeated the analysis of the G1/S checkpoint by BrdU staining with additional time points as requested (new Figure 5B). For simplicity, cells in S phase, G1 and G2 were plotted in individual diagrams. For the visualization of the gates used for the analysis representative FACS plots are shown in Supplemental Figure 6A,B. Experiments on MEFs have been moved to Supplemental Figure 6.

*Third, the panel on IR-induced phosphorylations (Figure 5C) should be extended by including different recovery times after IR, ranging from for example 5 minutes (early signaling) to 4 hours (sustained signaling) rather than showing a single time-point only.*

In response to this suggestion we repeated the Western blot analysis with earlier time-points. The new data is shown in Supplemental Fig. 6C.

*Finally, can the authors assess cellular survival / proliferation in response to IR of Obfc2b-deficient cells? Similar criteria apply also for the key experiments with the Obfc2b/Obfc2a double deficient cells.*

We were not sufficiently clear on this point. The analysis of proliferation was performed by CFSE labeling for B cells and can be found in Figure 4D and Supplemental Figure 5 for *Obfc2b.* For Obfc2a and Obfc2a/Obfc2b double deficient cells proliferation analysis has been performed in MEFs and is shown in Fig. 7D. We have tried to clarify this point in the text.

*2) On a more general note (linked to the previous point): As the authors outline already in the introduction, their findings are in conflict with proposed functions of SSB1 and SSB2 based on experiments in human cell lines by different labs. The authors should thus include a dedicated paragraph in the discussion to address what, in their opinion, can cause such differences and whether and how they could be reconciled.*

This is an excellent suggestion, we have added a dedicated paragraph to the discussion section.

*3) The authors suggest a (potentially redundant) function of Obfc2b/Obfc2a for cellular proliferation, and Obfc2a depletion results in reduced levels of p-Chk1 after IR, spontaneous accumulation of phosphorylated H2ax and in increased genomic aberrations. These observations are very interesting and could indicate that Obfc2b/Obfc2a are important to prevent replicative stress, possibly in a similar manner as the ssDNA binding protein Rpa1. Could the authors extend their analyses of this phenotype and test whether Obfc2b-, Obfc2a-, and Obfc2b/Obfc2a-deficient*

 

*cells are hypersensitive to experimentally induced replication stress, e.g. by addition of hydroxyurea or aphidicoline? Are ATR-dependent signaling and phosphorylation of downstream targets or Rpa1-loading in response to replicative stress affected? Is cellular survival in dose-response to hydroxyurea affected? Experiments in these directions could help better define this phenotype and even shed light on redundant versus*
*independent functions of Obfc2b and Obfc2a.*

This is an interesting point but our capacity to answer this question is limited by the fact that Obfc2a depleted cells show a proliferation arrest (Figure 7D), which could also cause reduced Chk1 phosphorylation after IR. We therefore analyzed *Obfc2a/hSSB2*-shRNA infected MEFs at day 3 post infection instead of at day 6. At day 3 the proliferation defect is not very pronounced. We then treated these cells with Hydroxyurea to induce Chk1 phosphorylation and we see no difference between Obfc2a deficient and proficient cells (Supplemental Figure 7D).

*4) A technical remark to the previous point: The observation that Obfc2a knockdown by shRNA causes spontaneous DNA damage (Fig. 7B) is intriguing but more controls should be provided to exclude that this increase in DDR signaling is not an unwanted byproduct of the shRNA treatment. For instance, the authors may knockdown Obfc2b and show that this does not increase γH2ax (consistently to what se see after genetic knockout).*

This is a good point. However, deficiency of either Obfc2b or Obfc2a can cause replication associated DNA damage as shown by γH2ax accumulation during embryogenesis (Figure 3C) and in MEFs (Figure 7B) respectively. We agree that the inclusion of another control shRNA would strengthen this finding but it would not answer the question of why we do not see the reported phenotype for hSSB1 and hSSB2 associated with initiation of the DDR.

*5) Fig. 5B should be supplemented with an appropriate control (ATM inhibitor, ATM -/- cells) to show that the G1/S checkpoint defect can be detected under these conditions.*

Thanks for pointing this out. ATMi is included in the new Figure 5B.

***Referee #2***

*The distribution of classical cartilage and bone markers (mentioned above) should be analyzed in Obfc2b deficient mice, on sections of limbs harvested at different stages of endochondral bone development (E14.4, E16.5, E18.5 for example).*

To determine whether *Obfc2b* deficiency affects the function of chondrocytes, osteoblasts or osteoclasts, we performed a microarray analysis on isolated chondrocytes, osteoblasts or osteoclasts from E18.5 embryos (Supplemental Figure 3A-C). In summary, we do not observe any difference in gene expression in the absence of Obfc2b suggesting that *Obfc2b* is dispensable for chondrocyte, osteoblast or osteoclast development. In agreement with this, we additionally show that *Obfc2b*$^{-/-}$ embryos exhibit a defect in mesenchymal condensations (see Figure 3D) that are required for bone formation and occur before the development of chondrocytes, osteoblasts and osteoclasts. These defects in condensations occur at the time point when we also see apoptosis (E12.5) in *Obfc2b*$^{-/-}$ embryos. We also see increased genomic damage in the tissues that develop skeletal defects at this time point (Figure 3C). We therefore postulate that tissue specific expression of *Obfc2b* represents a mechanism to avoid the risk of apoptosis arising from replication-associated DNA damage in highly proliferating cells, which in turn ensures the correct development of skeletal structures during early embryogenesis by (Figure 8).

*Better images illustrating the cleft palate defect could be provided (many examples of what people present can be found in published articles).*

We have made the requested changes in Figure 2.

*Cell proliferation (phospho-histone H3, for example), and cell death should be evaluated on sections of mutant and control limbs at various stages of skeletal development (E14.4, E16.5, E18.5). Quantifications of any relevant differences observed should be presented (with error bars*

*and p values). For all tissue samples, an adjacent section should be stained with H&E to show clearly the histology of control and mutant skeletal elements. TUNEL analyses should also be performed at stages earlier than E12.5 presented in Fig. 3A (E9.5, and/or E10.5 for example).*

This is a good point we have added representative images of TUNEL analysis at E10.5 and E16.5 to Supplemental Figure 4.

*Indeed, it is important to evaluate cell death in the limb ectoderm and limb mesoderm prior to chondrocyte differentiation. These observations could explain at least in part the missing digits and should be discussed accordingly. In fact, it could be interesting to analyze the effect of the loss of Cbfc2b on the expression of Shh and FGFs in the limb ectoderm since these molecules control autopod patterning, which is particularly affected in Cbfc2b deficient mice. TUNEL analyses should also be done in Cbf2b;p53 double KO mice to show that cell death is "rescued" in at least some skeletal elements of double mutant mice. The authors should not hesitate to blow up their pictures to better illustrate the cell death in the developing limb and elsewhere.*

Thanks for pointing this out the TUNEL images were enlarged and simplified for better understanding in Figure 3A and Supplemental Figure 4C.

*Additional comments to improve the quality of this manuscript:*

*- The discrepancy between what has been published about the role of hSSB1 in the DNA damage response, and the authors' data could be further discussed. The authors point out the fact that previous studies have been done in vitro, while they present in vivo data arguing against the conclusions of this former work. One can understand that results obtained in vivo may differ from what has been obtained in vitro, which may be less physiologically relevant. However, the authors also present several in vitro data, which differ from other in vitro results published previously. Is there any possibility to reconcile the old published data with their new data? It seems important to provide more elements of reflection to the readers, to understand why this works leads to different conclusions.*

This is an excellent suggestion, we have added a dedicated paragraph to the discussion section.

*- The authors could also discuss further why Obfc2b deficiency leads essentially to skeletal defects, while other tissues seem relatively intact. The compensatory increase of Obfc2a seems to occur in several tissues, including in ribs (Fig.6A), yet Obfc2a does not compensate for the loss of Obfc2b in this tissue. Is the ratio of Obfc2b/Obfc2a expression (physiological expression) different in skeletal tissues compared to other tissues? Would other genes compensate for the loss of Obfc2b in other tissues?*

This is an excellent question. We show that loss of *Obc2b* results in a compensatory increase of *Obfc2a* expression and to a minimal extend vice versa. This finding suggests that the presence of at least one of the two proteins, Obfc2b and Obfc2a, is crucial for normal cell function. However, some cells show a selective requirement for Obfc2b or Obfc2a as *Obfc2b*$^{-/-}$ E12.5 embryos still show Obfc2a expression and Obfc2a depleted MEFs still show Obfc2a expression.

*- The manuscript is overall not written well enough. This is particularly true for the abstract. For example, the authors write that Obfc2a and Obfc2b may have overlapping functions, and then write: "Consistent with this idea, we show that Obfc2b is not required for the initiation of DNA damage checkpoints and for maintenance of genomic stability in B lymphocytes and primary fibroblasts". It is hard to understand why it is consistent, not knowing what Obcfc2a function is. This sentence would actually suggest that Obcfc2a like Obcf2b is not required for the initiation of the DNA damage checkpoint and maintenance of genomic stability (if their functions are similar). What the authors mean, most likely, is that Obfc2a may play redundant functions with Obfc2b, and thus compensate for the loss of Obfc2b in deficient mice. This sentence makes the abstract confusing, especially since it opposes abruptly the first information mentioned in the abstract (hSSB1 is essential for the initiation of DNA damage checkpoint), without any explanation of this major discrepancy. The abstract should perhaps emphasize the physiological function of Cbfc2b, rather than the redundancy with Cbfc2a, which is too "central" in the current abstract.*

 

We have tried to modify the manuscript to increase clarity.

*- The titles of each paragraph in the result section are inappropriate, and/or inaccurate. For example, "increased skeletal apoptosis" sounds awkward because it is not clear what "skeletal apoptosis" exactly is. It is also not really accurate since apoptosis detected in the limb buds seems restricted to the limb ectoderm (in non skeletal tissue; see fig 3A). The title "Obfc2b is redundant in B lymphocytes and MEFs" is not particularly attractive since it does not say with what Obfc2c is redundant with. It is also not accurate since the content of this paragraph does not demonstrate that Obfc2b is redundant with anything, but rather dispensable for specific functions. This title also does not say anything about the role of this gene in the DNA damage response, which is the main point of this paragraph. Several additional titles are not particularly attractive, and could be modify to formulate precisely the conclusion of the data presented.*

The titles have been changed.

*- The manuscript is somewhat hard to read for people who are not familiar with the DNA damage response. Several acronyms are not spelled out, and the functions of several molecules are not described throughout the manuscript (gammaH2ax, ATM, MRN, CTIP, Ku80, Xrcc4, Lig4, etc.). Mentioning that gammaH2ax accumulates is meaningless to someone who does not know what it is.*

Thanks for pointing this out we have tried to clarify.

*- The rational for doing several experiments is not clearly presented in the manuscript.*

We tried to improve our introduction of each experiment.

*- Control (wild-type, or double heterozygous) littermates should be shown in figure 3B, where the skeletal phenotype of Obfc2b and p53 double deficient mice is presented.*

Images from Obfc2b$^{+/+}$p53$^{+/+}$ embryos have been included (Figure 3B).

***Referee #3***

*1. Text: SSB1 deficiency is not essential for ATM activation. This is an important message (since it contrasts the previous works; i.e. from KK Khanna or M Pagano) and should be emphasized in the abstract.*

Thanks for pointing this out. This information has been included in the abstract.

*2. Text: Skeletal (including skull) problems are also seeing in other genomic instability syndromes, including ATR mutations (Seckel Syndrome) that compromise the response to ssDNA. The authors could use this information for their discussion.*

This is a good point, we have added a paragraph to the discussion on this point.

*3. Experiment: The authors see TUNEL on the developing bone but, do they see DNA breaks and or an accumulation of single stranded DNA? It would be nice to look for H2AX-P and 53BP1 foci (for breaks), or RPA foci (for ssDNA). It would help to understand whether the skeletal defects are also related to genome maintenance, or a completely independent role of SSB1 in bone development.*

To address this issue we analyzed the hindlimbs and forelimbs of E12.5 embryos for γH2ax and p53 phosphorylation at serine 15. E12.5 is the time point where we also see apoptosis in the tissues that later develop the skeletal defects. Indeed, *Obfc2b/hSSB1* deficient embryos show an increase in γH2ax and p-p53, both of which are indicators of ongoing DNA damage. Therefore both, Obfc2b/hSSB1 and Obfc2a/hSSB2, seem to suppress replication associated DNA damage. However, the requirement of either seems to be highly cell type specific.

*4. Experiment: If one looks at the data presented in Fig. S3, it looks to me like SSB2 depleted cells*

 

*have normal levels of p53 phosphorylation, but reduced levels of Chk1 phosphorylation. If this is true, this is an important observation since it would suggest that SSB2 is important for ATR and not ATM activation. This is similar then to the role RPA -another ssDNA binding protein- in ATR activation. To clarify whether this is the case, I would suggest that the authors: (1) Look at Chk1 phosphorylation in response to hydroxyurea (which is a much better activator of ATR than IR) and (b) verify that replication rates are not lower on SSB2 depleted cells. If replication levels are lower, then it would be inconclusive to see lower levels of HU-induced Chk1-P (since this event occurs during replication).*

This is an interesting point but our capacity to answer this question is limited by the fact that Obfc2a depleted cells show a proliferation arrest (Figure 7D). The small reduction of p-Chk1 therefore rather reflects the decreased number of cells in S phase that would activate Chk1 through Atr. Indeed, if we treat MEFs infected with *Obfc2a*-shRNAs at an earlier time point with hydroxyurea where the proliferation arrest is less dramatic (day 3 after infection) we don't see a reduction in p-Chk1 levels (Supplemental Fig. 7D). We have tried to clarify this point in the revised text.

2nd Editorial Decision 08 August 2012

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by one of the original referees (see comments below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

You shall receive a formal letter of acceptance explaining all further proceedings and formalities shortly. In the meantime, should you not have emailed or faxed the respective publication licenses and page charge forms, please send them to our office at your earliest convenience.

Thank you once more for the opportunity to consider this nice work for publication, and please consider our journal again for submitting your most exciting work in the future!

Yours sincerely,

Hartmut Vodermaier, PhD
Senior Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

The Authors addressed my previous comments and included data that further strengthen their conclusions. I am happy to recommend this revised manuscript for publication. This is an exciting and in many ways provocative study and I expect that it would have a high impact in the field of DNA damage response.

 

 