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Ablation of Rassf2 induces bone defects and subsequent hematopoietic anomalies in mice

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1st Editorial Decision 07 July 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, referees 1 and 3 are overall more positive about the study, while referee 2 is significantly less enthusiastic, and does not support publication. All three referes do raise a number of important technical and conceptual concerns, but should you be able to address these in full, we would be willing to over-rule the negative recommendation of referee 2, and invite a revision on your manuscript.

The most critical points in our assessment are:
1. A better understanding of the apparently contradictory effects of Rassf2 on osteoclasts in vitro vs. in vivo.
2. Further data on NFkB pathway status and its effects on osteoblast differentiation downstream of Rassf2.
3. Demonstration of an endogenous interaction between Rassf2 and IKK.

If you have any questions or comments about the referees' reports and/or your revision, please don't hesitate to get in touch and I am happy to discuss this further.

I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. 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:

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We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your 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 for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

In their manuscript entitled "RASSF2 inhibits osteoclastic bone destruction and promotes osteoblastic bone formation," Song et al describe the phenotype of mice globally lacking RASSF2. They find that the mice become runted after birth, associated with severe osteopenia and bone marrow failure. Both osteoclasts and osteoblasts were reduced in vivo, although only the latter showed a cell autonomous defect in vitro that explained the in vivo phenotype. Mechanistically, RASSF2 was found to interact directly with IKK and inhibit it, in both osteoclasts and osteoblasts. Thus, RASSF2 ko cells showed exaggerated NF-kB signaling, which when inhibited restored the cell phenotypes to normal. Overall the manuscript is well written and most experiments are clearly presented. The finding that RASSF2-deficiency leads to bone marrow failure, most likely via NF-kB activation in the osteoblast, is novel and interesting. Some items need more clarification.

• In the results describing Fig 2 (p6) authors describe reduced chondroblasts and increased bone fragility. Neither of these aspects is visible or presented in the figure.
• How is it possible that the mice have "reactive follicular hyperplasia" in the spleen when they have a small spleen and reduced numbers of B and T cells?
• Since RASSF2 ko mice have increased osteoclasts in vitro there may be some direct effects of RASSF2 on myeloid cells, so the myeloid population should be examined along with B and T cells in bone marrow and spleen. In the reconstitution experiment in Fig 1E, the recipient mice should be examined in more detail to look at subsets of hematopoietic cells in bone marrow and periphery. This hematopoietic failure is a very novel finding and thus should be carefully supported. No previous connections between osteoblasts and hematopoiesis involving the Hippo or NF-kB pathway have been made. This effect on hematopoietic niche also could be mentioned in the title
• The number of osteoclasts in vivo is low but so is bone mass. Since cell autonomous differentiation is increased in the ko's, it is possible that the OCs that do form have increased activity. Activity of OC's in vitro (resorption on bone or dentin) should be analyzed. Without this data, the part of the title related to the osteoclasts (see above) does not make sense as the authors argue that in vivo OCs are decreased due to decreased RANKL. The phrase "promotes osteoclastic bone destruction" should not be included in the title.
• Data from microarrays is not presented clearly. The heat maps do not show wt vs ko data. Is the display a ratio of the two? I do not understand how to read the graphic in Fig 4D. All the dots are very close to the center except 2, and again there is no clear indication that the display represents a ratio of wt to ko.

Referee #2

This manuscript examined the role of RASSF2, a member of the Ras-association domain family (RASSF) proteins, in osteoblasts and osteoclasts in vitro and in bone. The authors showed that mice

lacking Rassf2 displayed early postnatal lethality (around 4 weeks old), which was associated with impaired hematopoiesis and a severe skeletal phenotype of osteoporosis. Ablation of Rassf2 significantly decreased both osteoblastogenesis and osteoclastogenesis in bone, although exogenously supplied RANKL-induced osteoclast differentiation was dramatically enhanced in bone marrow monocytes cultures from Rassf2 KO mice compared to that from wild-type mice. Furthermore, inactivation of Rassf2 impaired osteoblast differentiation. Finally, authors showed that Rassf2 associated with and inhibited IKK activity and that lack of Rassf2 activated the IKK/NF-κB signaling pathway, resulting in increased osteoclast differentiation. Based on these findings, the authors conclude that Rassf2 inhibits osteoclast-mediated bone resorption and promotes osteoblast-mediated bone formation.
Major concerns: Results from this study provide clear evidence that deletion of the Rassf2 gene significantly decreased bone mass. However, this study, in its current form, is still very preliminary, suffers from lack of rigor, and fails to provide convincing molecular mechanism(s) whereby Rassf2 modulates bone formation and resorption. First, since in vivo osteoclast differentiation was reduced in the KO bone probably due to reduced RANKL expression in osteoblasts, the effect of IKK/NF-κB activation caused by Rassf2 deficiency on in vivo osteoclast differentiation should be limited. Second, studies on the effects of Rassf2 and IKK-DN on osteoblast function should be more complete (see my specific comments below). And third, similar reduction in bone formation and bone resorption should result in a much less severe bone phenotype in the Rassf2 KO mice.

Specific comments:

1) Figure 2
a) Figure 2A, lack of statistics for the bone histomorphometry (μCT analysis).
b) Figure 2B, this result should be further confirmed by measuring the levels of serum CTX, RANKL, and OPG proteins, all in vivo indicators for osteoclast differentiation and bone resorption.
c) Figure 2C, it is surprising that the calcein-labeled bands were so sharp, which are usually very diffusive in rapidly growing bones (3 weeks of age). This concern will be greatly reduced if authors could show the whole images of the labeled sections. The authors should specify the detail how the MAR assay was performed (This reviewer could not find it in the Methods or Figure Legends).

2) Figure 3
a) Figure 3A, it will be interesting to determine if Rassf2 deficiency alters osteoclast bone-resorbing activity in vitro (i.e., to perform pit assay on dentin slices).
b) Figure 3B, it is important to determine if Rassf2 deficiency impairs the formation of osteoblast precursors (i.e., to perform CFU-OB assay in bone marrow cells). It is possible that Rassf2 plays a role in regulation of development of mesenchymal stem cells toward osteooblast precursors.

3) Figure 4
a) Figure 4A, the levels of both phospho- and total IκBα proteins were similarly upregulated during the time course. Authors need to use normalized data from multiple experiments and perform statistical analysis. It was not specified how many times these experiments were repeated.
b) Additional experiments are required to confirm the effects of Rassf2 overexpression on osteoblast differentiation parameters (e.g., expression levels of osteocalcin, bone sialoprotein, Runx2, osterix mRNAs and ALP activity)?
c) Does overexpression of Rassf2 increase RANKL/M-CSF expression in osteoblasts?
d) Figure 4 E and G, the authors need to show the total-IκBα in those two figure.

4) Figure 5
1) What is the functional significance of the Rassf2-IKK interaction and subsequent suppression of the IKK pathway in osteoclasts (Remember-in vivo osteoclast differentiation was reduced in Rassf2 KO bone)?
2) Figure 5B, the effect of Rassf2 on IKK phosphorylation was not impressive.

5) Figure 6
a) More experiments are needed to confirm the effects of IKK-DN on osteoblast differentiation parameters (e.g., expression levels of osteocalcin, bone sialoprotein, Runx2, osterix mRNA and ALP activity)?
b) Does overexpression of IKK-DN increase RANKL/M-CSF expression in osteoblasts?
c) Figure 6E (working model), authors missed an important part-Rassf2 increases RANKL

expression, which favors osteoclast differentiation.

6) Figure S3
Figure S3C, it is not clear why the expression of Rassf2 protein was decreased during osteoclast differentiation.

7) Figure S4
1) Data from this figure is critical to support an important role for Rassf2 in regulating osteoblast differentiation. This data should be presented in a regular figure rather than in a supplementary figure.
2) Figure S4B, the level of OPG should be included.

8) Figure S5
Lack of total IκBα in Figure S5 A and B

Referee #3

The authors demonstrate that systemic deletion of RASSF2 induces both hematopoietic and osteoporotic phenotype in RASSF2KO mice. They also show that the hematopoietic phenotype in the RASSF2KO mice is most likely due to the microenvironmental changes due to the impairment of proper osteogenesis. Furthermore, they found that the lack of RASSF2 deregulates NF-kB signaling in osteoblasts, which in turn induces impairment of osteoblast differentiation and decreases in osteoblasts and osteoclasts.

Specific
In Figure 3C, the authors show that RASSF2 regulates differentiation of BMM through secretion of factors from osteoblasts. They show that some factors are downregulated in RASSF2KO mice. Here, it would be ideal to show that production of these factors is negatively regulated by NF-kB in osteoblasts.

Although the authors show that RASSF2 KO markedly stimulates IkB phosphorylation in osteoblasts, they do not show that NF-kB is really activated in osteoblasts.

In Figure 5, the authors propose that RASSF2 inhibits IKK through direct physical interaction. Here, such mechanism is shown only with overexpression. It would be important to show that such interaction occurs in the physiological context.

In Figure 6C, the phospho-IkB is shown as a doublet in SDS-PAGE gel. Although the upper band is stronger in WT, the lower band is stronger in RASSF2KO. Please explain why.

Minor,
Please explain more regarding Figure 4D in the text. Explanation is missing.

Please explain why the NF-kB pathway was not affected in BMMs at baseline (time 0) in Figure 4E.

In page 10, lines 2 and 5, Figure 2C should be read as Figure 3C.

1st Revision - authors' response 04 November 2011

Response to referee comments (EMBOJ-2011-78319)

Referee #1

General comment: *In their manuscript entitled "RASSF2 inhibits osteoclastic bone destruction and promotes osteoblastic bone formation," Song et al describe the phenotype of mice globally lacking RASSF2. They find that the mice become runted after birth, associated with severe osteopenia and bone marrow failure. Both osteoclasts and osteoblasts were reduced in vivo, although only the latter showed a cell autonomous defect in vitro that explained the in vivo phenotype. Mechanistically,*

RASSF2 was found to interact directly with IKK and inhibit it, in both osteoclasts and osteoblasts. Thus, RASSF2 KO cells showed exaggerated NF-kB signaling, which when inhibited restored the cell phenotypes to normal. Overall the manuscript is well written and most experiments are clearly presented. The finding that RASSF2-deficiency leads to bone marrow failure, most likely via NF-kB activation in the osteoblast, is novel and interesting. Some items need more clarification.
Answer: We thank the reviewer for the critical and positive comments. In response, we have performed the additional experiments that are described in the revised manuscript.

Comment 1: *In the results describing Fig 2 (p6) authors describe reduced chondroblasts and increased bone fragility. Neither of these aspects is visible or presented in the figure.*
Answer: We agree with the reviewer's criticism. We have changed the previous sentence to "Moreover, *Rassf2*$^{-/-}$ animals also exhibited marked abnormalities in bone development, including decreases in bone mass and the extent of trabecular bone (Figure 2A and B)".

Comment 2: *How is it possible that the mice have "reactive follicular hyperplasia" in the spleen when they have a small spleen and reduced numbers of B and T cells?*
Answer: A histological definition of acute reactive hyperplasia in the spleen is as follows: "Variable degrees of congestion, diffuse immunoblastic and plasmacytic proliferation, and outpouring of neutrophils in the red pulp" (Reference. Rosai and Ackerman's surgical pathology, Juan Rosai, 9th edition, vol.2, pp.2022-2023). Acute reactive hyperplasia shows more pathological variation than does chronic hyperplasia. Based on the histological definition, reactive follicular hyperplasia, as the term is used in our manuscript, does not imply that splenomegaly was present, but rather that germinal centers were histologically evident in the white pulp, and mild congestion in the red pulp, of *Rassf2*$^{-/-}$ spleens. We have changed our description to: "mild degrees of congestion in the red pulp of *Rassf2*$^{-/-}$ spleen".

Comment 3: *Since RASSF2 KO mice have increased osteoclasts in vitro there may be some direct effects of RASSF2 on myeloid cells, so the myeloid population should be examined along with B and T cells in bone marrow and spleen.*
Answer: As suggested, we have examined the myeloid-/B- and T-cell populations in bone marrow and spleen and present our data in Supplementary Figure S2C.

Comment 4: *In the reconstitution experiment in Fig 1E, the recipient mice should be examined in more detail to look at subsets of hematopoietic cells in bone marrow and periphery.*
Answer: As suggested, we have added new data on the levels of donor-derived subpopulations of hematopoietic cells in both the bone marrow and peripheral blood of recipient mice; the results are shown in Supplementary Figure S3.

Comment 5: *This hematopoietic failure is a very novel finding and thus should be carefully supported. No previous connections between osteoblasts and hematopoiesis involving the Hippo or NF-kB pathway have been made. This effect on hematopoietic niche also could be mentioned in the title.*
Answer: We appreciate the reviewer's thoughtful comments. However, we feel that our data do not offer sufficiently strong evidence that either the Hippo or NF-kB pathway plays a critical role in connecting osteoblast development and hematopoiesis. Thus, we would like to change our title to "Ablation of Rassf2 induces the bone defects and subsequent hematopoietic anomalies in mice". We hope the new title is appropriate.

Comment 6: *The number of osteoclasts in vivo is low but so is bone mass. Since cell autonomous differentiation is increased in the ko's, it is possible that the OCs that do form have increased activity. Activity of OC's in vitro (resorption on bone or dentin) should be analyzed. Without this data, the part of the title related to the osteoclasts (see above) does not make sense as the authors argue that in vivo OCs are decreased due to decreased RANKL. The phrase "promotes (misprint: inhibits?) osteoclastic bone destruction" should not be included in the title.*
Answer: As suggested, we have now performed an assay measuring osteoclast functionality. As shown in Figure 3B, the resorbed area of pits formed on dentine slices was markedly increased when *Rassf2*-deficient osteoclasts were employed, compared to wild-type control cells. We have added details of our method to Materials and Methods. We also show that *Rassf2*$^{-/-}$ mice had lower levels of serum M-CSF and RANKL than did wild-type littermates (Figure 2D). This is a consequence of the lower numbers of osteoblasts in trabecular bone (Figure 2B), and reduced T-cell populations in

the thymus, spleen, peripheral blood, and bone marrow (Figure 1C and Supplementary Figure S2C). Thus, we believe that, *in vivo*, osteoclast numbers are decreased principally because of defects in both osteoblasts *per se* and in immune cells that express osteoclastogenic factors (M-CSF and RANKL).

Comment 7: *Data from microarrays is not presented clearly. The heat maps do not show WT vs KO data. Is the display a ratio of the two? I do not understand how to read the graphic in Fig 4D. All the dots are very close to the center except 2, and again there is no clear indication that the display represents a ratio of WT to KO.*
Answer: We agree with the reviewer's comment. To clarify the matter, we have added a sentence: "Data are expressed relative to the values for wild-type cells". Also, the descriptions in the Figure Legends have been expanded (Figure 3D, and Figure 4C and D). We have changed the format of Figure 4D to a heatmap, as shown now in Figure 4C.

Referee #2

General comment: *This manuscript examined the role of RASSF2, a member of the Ras-association domain family (RASSF) proteins, in osteoblasts and osteoclasts in vitro and in bone. The authors showed that mice lacking Rassf2 displayed early postnatal lethality (around 4 weeks old), which was associated with impaired hematopoiesis and a severe skeletal phenotype of osteoporosis. Ablation of Rassf2 significantly decreased both osteoblastogenesis and osteoclastogenesis in bone, although exogenously supplied RANKL-induced osteoclast differentiation was dramatically enhanced in bone marrow monocytes cultures from Rassf2 KO mice compared to that from wild-type mice. Furthermore, inactivation of Rassf2 impaired osteoblast differentiation. Finally, authors showed that Rassf2 associated with and inhibited IKK activity and that lack of Rassf2 activated the IKK/NF-kB signaling pathway, resulting in increased osteoclast differentiation. Based on these findings, the authors conclude that Rassf2 inhibits osteoclast-mediated bone resorption and promotes osteoblast-mediated bone formation.*
Major concerns: Results from this study provide clear evidence that deletion of the Rassf2 gene significantly decreased bone mass. However, this study, in its current form, is still very preliminary, suffers from lack of rigor, and fails to provide convincing molecular mechanism(s) whereby Rassf2 modulates bone formation and resorption.

First, since in vivo osteoclast differentiation was reduced in the KO bone probably due to reduced RANKL expression in osteoblasts, the effect of IKK/NF-kB activation caused by Rassf2 deficiency on in vivo osteoclast differentiation should be limited. Second, studies on the effects of Rassf2 and IKK-DN on osteoblast function should be more complete (see my specific comments below). And third, similar reduction in bone formation and bone resorption should result in a much less severe bone phenotype in the Rassf2 KO mice.
Answer: We have done our best to deal with all reviewer comments in the revised manuscript. First, we agree with the comments made above by the reviewer. Osteoclast formation is determined by the balance between osteoblast-produced stimulators and inhibitors. To address this topic, we have performed additional experiments including measurement of the levels of osteoclast-regulating factors (RANKL, M-CSF, and OPG) in serum and in the culture medium of osteoblasts, and we have assessed the bone resorption capacity of osteoclasts. Second, and again in response to the reviewer's comment, we have performed an additional experiment confirming the rescue of osteoclastic and osteoblastic marker gene expression by reintroduction of *Rassf2* or *IKK-DN*. Third, reduction in bone mass depends on various factors, including excessive bone destruction by osteoclasts relative to bone formation by osteoblasts; increased bone resorption by osteoclasts, decreased bone synthesis by osteoblasts, and decreased osteoclast and osteoblast formation *per se*. In the present study, the osteoporotic phenotype of *Rassf2*$^{-/-}$ mice may be attributable to reduced bone turnover resulting from impairment in osteoblast and osteoclast formation. Collectively, the reduction in bone mass caused by low-level osteoclast and osteoblast formation may be further exacerbated by an increase in bone resorption by osteoclasts relative to bone formation by osteoblasts. We hope that when the manuscript is read as a whole, the reviewer will accept the rationale of our work.

Comment 1: 1-1; *In Figure 2A, lack of statistics for the bone histomorphometry (mCT analysis).* ***1-2;*** *Figure 2B, this result should be further confirmed by measuring the levels of serum CTX,*

RANKL, and OPG proteins, all in vivo indicators for osteoclast differentiation and bone resorption. ***1-3;*** *Figure 2C, it is surprising that the calcein-labeled bands were so sharp, which are usually very diffusive in rapidly growing bones (3 weeks of age). This concern will be greatly reduced if authors could show the whole images of the labeled sections. The authors should specify the detail how the MAR assay was performed (This reviewer could not find it in the Methods or Figure Legends).*
Answer 1-1: As suggested, we have revised the mode of presentation of statistical data in the entire manuscript.
Answer 1-2: As suggested, we have newly measured the levels of M-CSF, RANKL, and OPG in serum, and those of CTX in serum and urine. *Rassf2*-deficient mice showed decreased serum levels of M-CSF and RANKL compared with those of wild-type littermates, but OPG levels did not differ between the two strains. These results indicate that a decrease in M-CSF and RANKL synthesis by osteoblast cell lineages and activated immune T-cells causes a defect in osteoclast formation. In addition, we observed that the levels of CTX in both serum and urine were significantly decreased in *Rassf2*-deficient mice compared to control littermates; this was attributable to low-level bone turnover caused by defective osteoblast and osteoclast formation in *Rassf2*-deficient animals. We have added new data to the panel of Figure 2 and we describe our results in the "Results" and "Materials and Methods" sections.
Answer 1-3: We agree with the reviewer's comment. Calcein-labeled bands tend to be diffuse in growing bone. Additionally, the intensity of such bands is determined by calcein dosage as well as the duration of treatment and the interval that elapses after the final treatment. Thus, we have described our method, in detail, in "Materials and Methods", and hope that our explanation is convincing.

Comment 2: 2-1; *In Figure 3A, it will be interesting to determine if Rassf2 deficiency alters osteoclast bone-resorbing activity in vitro (i.e., to perform pit assay on dentin slices).* ***2-2;*** *In Figure 3B, it is important to determine if Rassf2 deficiency impairs the formation of osteoblast precursors (i.e., to perform CFU-OB assay in bone marrow cells). It is possible that Rassf2 plays a role in regulation of development of mesenchymal stem cells toward osteooblast precursors.*
Answer 2-1: We appreciate the reviewer's thoughtful comments. As suggested, we have now measured osteoclast bone-resorbing activity *in vitro*. We found that the resorbed area of pits on dentine slices was markedly increased when *Rassf2*-deficient osteoclasts, compared to wild-type control cells, were used (Figure 3B). We have added the details of our method to the "Materials and Methods" section.
Answer 2-2: We also performed a CFU-OB assay using bone marrow cells. When such cells, prepared from 2 week-old wild type and *Rassf2*$^{-/-}$ mice, were cultured in osteogenic medium containing ascorbic acid and b-glycerophosphate, we observed no difference in the extent of colony formation. Thus, we believe that the lack of *Rassf2* did not affect the formation of mesenchymal stem cell-derived osteoblast progenitors. This observation has now been added to the legend of Supplementary Figure S4.

Comment 3: 3-1; *In Figure 4A, the levels of both phospho- and total IkBa; proteins were similarly upregulated during the time course. Authors need to use normalized data from multiple experiments and perform statistical analysis. It was not specified how many times these experiments were repeated.* ***3-2;*** *Additional experiments are required to confirm the effects of Rassf2 overexpression on osteoblast differentiation parameters (e.g., expression levels of osteocalcin, bone sialoprotein, Runx2, osterix mRNAs and ALP activity)?* ***3-3;*** *Does overexpression of Rassf2 increase RANKL/M-CSF expression in osteoblasts?* ***3-4;*** *Figure 4 E and G, the authors need to show the total-IkBa; in those two figure.*
Answer 3-1: The data of Figure 4A and B are representative of those of three independent experiments. As band intensity revealed by immunoblotting is difficult to analyze statistically, we now show the data as follows: The band intensity of phosphorylated IkBa is normalized to the density of total IkBa, after which the extent of phosphorylated IkBa is represented as a -fold-induction relative to the control value.
Answer 3-2 and 3-3: As suggested, we have now performed an additional experiment to confirm the rescue of osteoclastic and osteoblastic marker gene expression by *Rassf2* restoration to *Rassf2*-null cells. Real-time PCR showed that expression of osteoclastogenic (TRAP [encoded by *Acp5*]; and *Nfatc1*) and osteoblastogenic gene markers (*Runx2*; osterix [encoded by *Sp7*]; osteocalcin [encoded by *Bglap1*]; *Rankl*; and M-CSF [encoded by *Csf1*]) upon RASSF2 reintroduction was rescued to levels comparable to those of wild-type cells (Supplementary Figure S9). Notably,

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overexpression of RASSF2 in wild-type primary cells normally induces high-level cell death. Thus, we could not overexpress RASSF2 in such primary cells.
Answer 3-4: As suggested, we have added the immunoblot data on total IkBa levels to the panels of Figure 4E and G.

Comment 4: 4-1; *In Figure 5, what is the functional significance of the Rassf2-IKK interaction and subsequent suppression of the IKK pathway in osteoclasts (Remember-in vivo osteoclast differentiation was reduced in Rassf2 KO bone)?* ***4-2;*** *Figure 5B, the effect of Rassf2 on IKK phosphorylation was not impressive.*
Answer 4-1: *Rassf2*$^{-/-}$ BMMs more readily differentiated into osteoclasts than did wild- type BMMs in the presence of M-CSF and RANKL *in vitro*. However, *in vivo*, the numbers of osteoclasts in trabecular bone tissue were lower in *Rassf2*$^{-/-}$ mice, because osteoblast function was impaired. Also, the expression levels of M-CSF and RANKL were reduced *in vivo*. It is well-known that efficient osteoclast formation is achieved by a delicate balance between RANKL-induced upregulating stimulators and downregulating inhibitors. Upon osteoclast differentiation, numerous upregulated factors predominantly act as stimulators whereas downregulated effectors, including Id helix-loop-helix proteins, MafB, and interferon regulatory factor-8, serve as inhibitors of the process (Lee J. et al., 2006, Blood 107:2686-2693; Kim K. et al., 2007, Blood 109:3253-3259; Zhao B. et al., 2009, Nature Medicine 15:1066-1071). In our present study, RANKL-induced upregulation of *Rassf2* paradoxically served to inhibit osteoclast formation via inactivation of NF-kB signaling. We have discussed this fact in Results.
Answer 4-2: We have performed the *in vitro* kinase assay for many years. Thus, we are convinced that a marked reduction in GST-IkBa substrate phosphorylation by IKK was evident in the presence of RASSF2 (Figure 5C). The immunoprecipitation kinase assay and the *in vitro* kinase assay data (Figure 5C and D) are each representative of those of three independent experiments, and clearly support an inhibitory role for RASSF2 in terms of IKK action. To further confirm inhibition by RASSF2 of IKK-NFkB signaling, we have performed new subcellular fractionation and immunostaining experiments and have detected both enhanced IkBa phosphorylation and degradation (Figure 4), and nuclear translocation of NF-kB, in *Rassf2*$^{-/-}$ osteoblast precursors (Figure 5F and G).

Comment 5: 5-1; *In Figure 6, More experiments are needed to confirm the effects of IKK-DN on osteoblast differentiation parameters (e.g., expression levels of osteocalcin, bone sialoprotein, Runx2, osterix mRNA and ALP activity)?* ***5-2;*** *Does overexpression of IKK-DN increase RANKL/M-CSF expression in osteoblasts?* ***5-3;*** *Figure 6E (working model), authors missed an important part; Rassf2 increases RANKL expression, which favors osteoclast differentiation.*
Answer 5-1 and 5-2: As suggested by the reviewer, we have performed an additional experiment to confirm the rescue of osteoclastic and osteoblastic marker genes upon IKK-DN introduction. Real-time PCR showed that *Rassf2*$^{-/-}$ cells overexpressing IKK-DN were rescued in terms of expression of both osteoclastogenic (TRAP [encoded by *Acp5*]; and *Nfatc1*) and osteoblastogenic gene markers (osterix [encoded by *Sp7*]; *Rankl*; and M-CSF [encoded by *Csf1*]). The expression levels became similar to those of wild-type cells (Supplementary Figure S10).
Answer 5-3: We welcome the reviewer's suggestion and have modified Figure 6E.

Comment 6: *In Figure S3C, it is not clear why the expression of Rassf2 protein was decreased during osteoclast differentiation.*
Answer: Although the levels of *Rassf2* expression during osteoclast differentiation differed slightly among experiments, in most instances, both the level of mRNA encoding the protein, and that of Rassf2 *per se*, gradually increased during such differentiation. We have added a new relevant photograph to Supplementary Figure S5C.

Comment 7: 7-1; *In Figure S4, data from this figure is critical to support an important role for Rassf2 in regulating osteoblast differentiation. This data should be presented in a regular figure rather than in a supplementary figure.* ***7-2;*** *In Figure S4B, the level of OPG should be included.*
Answer 7-1: As suggested, we have moved the Figure S4 data into the principal Figure 3.
Answer 7-2: We have added new data on the level of mRNA encoding OPG (the relevant gene is *Tnfrsf11b*) to Figure 3E.

Comment 8: *In Figure S5, Lack of total IkBa; in Figure S5 A and B*

Answer: We have added immunoblot data on total IkBa levels to the panels of Supplementary Figure 7A and B.

Referee #3

General comment: *The authors demonstrate that systemic deletion of RASSF2 induces both hematopoietic and osteoporotic phenotype in RASSF2 KO mice. They also show that the hematopoietic phenotype in the RASSF2 KO mice is most likely due to the microenvironmental changes due to the impairment of proper osteogenesis. Furthermore, they found that the lack of RASSF2 deregulates NF-kB signaling in osteoblasts, which in turn induces impairment of osteoblast differentiation and decreases in osteoblasts and osteoclasts.*
Answer: We deeply appreciate the reviewer's interest and the helpful comments.

Comment 1: *In Figure 3C, the authors show that RASSF2 regulates differentiation of BMM through secretion of factors from osteoblasts. They show that some factors are downregulated in RASSF2 KO mice. Here, it would be ideal to show that production of these factors is negatively regulated by NF-kB in osteoblasts.*
Answer: We appreciate the reviewer's thoughtful suggestions. We have now shown that the mRNA levels encoding M-CSF and RANKL, and the levels of the relevant proteins, expressed in osteoclasts and stimulating osteoclast formation, are downregulated in *Rassf2*-deficient osteoblasts (Supplementary Figure S6, and Figure 3E and H). We also observed that consistent activation of NF-kB in *Rassf2*-deficient osteoblasts induced downregulation of NF-kB-dependent target genes (*Ppard, Htra3, Adrb2, Snrpd3, Gsdm2,* and *Tbc1d5*), both before and during differentiation, but the roles of these genes in terms of osteoblast differentiation remain unknown (Supplementary Figure S8). We now describe the conditions for downregulation of NF-kB-dependent target genes in Results.

Comment 2: *Although the authors show that RASSF2 KO markedly stimulates IkB phosphorylation in osteoblasts, they do not show that NF-kB is really activated in osteoblasts.*
Answer: This is an excellent suggestion. To show that NF-kB is really activated in osteoblasts, we performed subcellular fractionation and immunostaining analysis and confirmed increased nuclear translocation of NF-kB in *Rassf2*$^{-/-}$ osteoblast precursors (Figure 5F and G).

Comment 3: *In Figure 5, the authors propose that RASSF2 inhibits IKK through direct physical interaction. Here, such mechanism is shown only with overexpression. It would be important to show that such interaction occurs in the physiological context.*
Answer: This was the most challenging aspect of the revision. We made many efforts to show an endogenous interaction between RASSF2 and IKK. However, we found that no commercially available antibody against IKKa, IKKb, and RASSF2 quantitatively immunoprecipitated the target protein. Therefore, we could not detect endogenous association in mouse osteoblast precursor cells. To overcome this technical problem, a retroviral vector encoding Flag-tagged RASSF2 was stably introduced into *Rassf2*$^{-/-}$ osteoblast precursors. Now, we could show that a small proportion of endogenous IKKa and IKKb co-precipitated with Flag-RASSF2 (Figure 5B) from RASSF2-complemented cells. These data are described in the revised manuscript.

Comment 4: *In Figure 6C, the phospho-IkB is shown as a doublet in SDS-PAGE gel. Although the upper band is stronger in WT, the lower band is stronger in RASSF2 KO. Please explain why.*
Answer: When we carefully compared the location of phospho-IkBa and degraded IkBa, in a side-by-side manner, the lower band was the true phospho-IkB. Sometimes, a nonspecific upper band was evident when proteins from osteoblastic cell types were immunoblotted with anti-phospho-IkBa.

Comment 5: *Please explain more regarding Figure 4D in the text. Explanation is missing.*
Answer: We agree with the reviewer's comment. To clarify the matter, we have added a sentence: "Data are expressed relative to the values for wild-type cells". Also, the descriptions in the Figure Legends have been expanded (Figure 3D, and Figure 4C and D). We have changed the format of Figure 4D to a heatmap, as now shown in Figure 4C.

Comment 6: *Please explain why the NF-kB pathway was not affected in BMMs at baseline (time 0) in Figure 4E.*

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Answer: The reviewer asks why the basal activity of IkBa in *Rassf2*-deficient BMMs differs from that in *Rassf2*-deficient osteoblast precursors. Overall, compared with wild- type cells, *Rassf2*$^{-/-}$ osteoblast precursors showed enhanced basal activity of NF-kB signals, characterized by increased levels of phosphorylated IkBa and a greater level of IkBa degradation. However, the basal activity of NF-kB did not differ between wild-type and *Rassf2*$^{-/-}$ BMMs. We believe that this reflects a fundamental difference in the nature of the signal networks in the two cell types.

Comment 7: *In page 10, lines 2 and 5, Figure 2C should be read as Figure 3C.*
Answer: Thank you for highlighting this mistake. Figure 2C has been corrected to read Figure 3C in the revised manuscript.

2nd Editorial Decision 25 November 2011

Thank you for the submission of your revised manuscript to The EMBO Journal. It has been sent to two of the original reviewers that now consider that all their concerns have been properly addressed and your manuscript is ready for publication.

However, as you will see below referee #1 still points out to a number of minor details that need your attention before your manuscript can be officially accepted. I would like to make clear nevertheless that the re-organization of the Results section would not be necessary, although it could certainly benefit from splitting into further subsections.

Do not hesitate to contact me in case you have any further questions.
I am looking forward to seeing the revised, final version of your manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

Overall, the authors have put a lot of work into improving this manuscript and answering the reviewers concerns. Overall the data are solid and represent an important description of a new regulator of bone and hematopoiesis. The remaining issues with the manuscript are purely editorial, to address places where the manuscript is not as clear as possible.

It is not clear from this abstract that hematopoietic defects are secondary to bone changes, and that OB defects determine in vivo phenotype.

In the introduction, the comment that "Mesenchymal stem cell (MSC)-derived osteoblasts and HSC-derived osteoclasts can communicate via diffusible paracrine factors, including receptor activator of NF-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF)" is not accurate since a large fraction of both of these factors are expressed in membrane-bound forms in OBs.

In the end, the weakest mechanistic link in this paper is the connection between Rassf2 -/- osteoblast defect and the hematopoietic defect since the authors were unable to transplant WT marrow into KO mice to examine the supportive microenvironment, and they don't examine any features of osteoblasts that are specifically related to differentiation of any cells other than osteoclasts. Thus, the organization of the paper, with the transition from hematopoiesis to bone cells " We then focused on microenvironmental changes that might explain the hematopoietic defects and growth retardation of Rassf2−/− mice" indicating that the link is going to be elucidated does not seem logical. Also, since ultimately the authors conclude that the osteoblast defects (and not the enhanced osteoclastogenesis seen with isolated myeloid cells) are most important for the in vivo phenotype, the presentation of the data in Fig 3 does not convey this so clearly. Yes, the in vitro osteoclast differentiation should be shown, but the order of the data and its description in the text does not lead

to the conclusions in the most logical manner. Overall, many of the paragraphs in the results section are now very long, and could be broken up for clarity.

It is not clear what is meant by the statement "We also observed that consistent activation of NF-κB in Rassf2-deficient osteoblasts induced downregulation of NF-κB-dependent target genes (Ppard, Htra3, Adrb2, Snrpd3, Gsdm2, and Tbc1d5) both before and during differentiation (Supplementary Figure S8)."
Rassf2-/- osteoblasts have higher NF-kB activation so downregulation of NF-kB dependent targets should not be observed. If these genes are supposed to be downregulated by NF-kB, then this should be stated clearly.

Referee #3

First of all, I apologize for my delay in returning this report.
I found that the authors addressed all issues that I raised and the paper is improved significantly. I believe that the paper provides the field with conceptually novel and important information. I strongly support publication of this manuscript in the Journal.

2nd Revision - authors' response 05 December 2011

Referee #1

General comment: *Overall, the authors have put a lot of work into improving this manuscript and answering the reviewers concerns. Overall the data are solid and represent an important description of a new regulator of bone and hematopoiesis. The remaining issues with the manuscript are purely editorial, to address places where the manuscript is not as clear as possible.*
Answer: We thank the reviewer for the helpful comments. As suggested, we made some modifications to improve the manuscript.

Comment 1: *It is not clear from this abstract that hematopoietic defects are secondary to bone changes, and that OB defects determine in vivo phenotype.*
Answer: To clarify this point that hematopoietic defects are secondary to bone changes, and that osteoblast defects determine in vivo phenotype, we have modified the sentence as follows in the abstract. "an intrinsic defect in osteoblast differentiation from *Rassf2*$^{-/-}$ osteoblast precursors likely leads to both hematopoiesis and osteoclast defects in *Rassf2*$^{-/-}$ mice".

Comment 2: *In the introduction, the comment that "Mesenchymal stem cell (MSC)-derived osteoblasts and HSC-derived osteoclasts can communicate via diffusible paracrine factors, including receptor activator of NF-kB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF)" is not accurate since a large fraction of both of these factors are expressed in membrane-bound forms in OBs.*
Answer: We agree with the reviewer's comment. To avoid this ambiguity, we have modified the previous sentence to "Mesenchymal stem cell (MSC)-derived osteoblasts and HSC-derived osteoclasts can communicate via paracrine factors, including receptor activator of NF-kB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF)".

Comment 3: *In the end, the weakest mechanistic link in this paper is the connection between Rassf2-/- osteoblast defect and the hematopoietic defect since the authors were unable to transplant WT marrow into KO mice to examine the supportive microenvironment, and they don't examine any features of osteoblasts that are specifically related to differentiation of any cells other than osteoclasts. Thus, the organization of the paper, with the transition from hematopoiesis to bone cells "We then focused on microenvironmental changes that might explain the hematopoietic defects and growth retardation of Rassf2-/- mice" indicating that the link is going to be elucidated does not seem logical.*
Answer: I agree with the reviewer's concern, in principle. To depict the logical connection between *Rassf2*$^{-/-}$ osteoblastic and hematopoietic defect, we have changed the previous sentence to "Since it

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is known that the activation of osteoblasts in the bone marrow regulates the HSC niche (Calvi LM et al, 2003, Nature 425: 841-846), we then focused on bone microenvironment changes that might explain the hematopoietic defects and growth retardation of *Rassf2*$^{-/-}$ mice"

Even though we were practically unable to transplant WT marrow into KO mice, we showed that the transplanted mutant BM cells in WT mice exhibited normal hematopoietic developmental capacity (Figure 1E and Supplementary Figure S3). Based on this result, we at least thought that the impaired hematopoiesis of *Rassf2*$^{-/-}$ mice was probably not attributable to an intrinsic developmental defect of HSCs. In fact, we also discussed this concern as follows in the discussion: "However, we cannot currently exclude the possibility that *Rassf2* deficiency causes intrinsic defects in lymphoid organ development, immune cell differentiation, or maintenance of HSCs or MSCs. Therefore, in light of the fact that crosstalk occurs between the immune and skeletal system of *Rassf2*$^{-/-}$ mice, tissue-specific depletion of *Rassf2* will be required to identify, in more detail, the roles played by RASSF2 during hematopoietic development and bone remodeling."

Comment 4: *Also, since ultimately the authors conclude that the osteoblast defects (and not the enhanced osteoclastogenesis seen with isolated myeloid cells) are most important for the in vivo phenotype, the presentation of the data in Fig 3 does not convey this so clearly. Yes, the in vitro osteoclast differentiation should be shown, but the order of the data and its description in the text does not lead to the conclusions in the most logical manner.*
Answer: We welcome the reviewer's thoughtful suggestion. To clearly emphasize on osteoblast defects as *in vivo* phenotypes, we have moved the osteoblast defect data from Figure 3C-F to Figure 3A-D, and changed the descriptions in text accordingly. On this account, we also have switched the order of Supplementary Figure S5 and S6.

Comment 5: *Overall, many of the paragraphs in the results section are now very long, and could be broken up for clarity.*
Answer: Even though we also knew that many of paragraphs are relatively long, we think that these paragraphs are needed to fully explain to the link and help the readers' understanding. We would appreciate it if the reviewer would reconsider this point.

Comment 6: *It is not clear what is meant by the statement "We also observed that consistent activation of NF-kB in Rassf2-deficient osteoblasts induced downregulation of NF-kB-dependent target genes (Ppard, Htra3, Adrb2, Snrpd3, Gsdm2, and Tbc1d5) both before and during differentiation (Supplementary Figure S8)."Rassf2-/- osteoblasts have higher NF-kB activation so downregulation of NF-kB dependent targets should not be observed. If these genes are supposed to be downregulated by NF-kB, then this should be stated clearly.*
Answer: We appreciate the reviewer's excellent point about this ambiguity. To avoid this ambiguous description, we have changed the sentence as follows: "We also observed that some of putative NF-kB target genes such as *Ppard*, *Htra3*, *Adrb2*, *Snrpd3*, *Gsdm2*, and *Tbc1d5* were downregulated in *Rassf2*-deficient osteoblasts but upregulated in *Rassf2*-deficient osteoclasts, both before and during differentiation. These data suggested that a subset of known NF-kB target genes could be differentially regulated in the context of osteoblasts and osteoclasts (Supplementary Figure S8). However, the roles played by these genes in osteoblast and osteoclast differentiation are not yet known".

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