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Shp2 Signaling is Essential to the Suppression of Senescence in PyMT-induced Mammary Gland Cancer in Mice

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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 20 June 2014

Thank you for submitting your manuscript to The EMBO Journal. Three referees have now seen your study and their comments are provided below.

As you can see, the referees find the analysis interesting and suitable for publication here. They raise a number of specific issues that should be addressed before publication here. In particular the microarray and bioinformations analysis needs to be better described, a better rationale for why you chose to focus on Skp2, Aurka, Dll1 and Hey1 and more data to support the role of these players in the observed senescence phenotype.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, that addresses the concerns raised in full. I should add that it is EMBO Journal policy to allow a single major round of revision and that it is therefore important to sort out the raised issues at this stage.

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://emboj.embopress.org/about#Transparent_Process

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.

REFEREE REPORTS

Referee #1:

In this manuscript entitled "Shp2 signaling is essential to the suppression of senescence in mammary gland cancer in mice" the authors provide evidence that the genetic ablation or pharmacological blockage of Shp2 inhibits cell proliferation and the self-renewal capacity of breast PyMT tumour cells. The Shp2 ablation and inhibition correlates with the induction of cellular senescence, and Shp2-regulated gene expression programs predict the survival of breast cancer patients. In addition, they propose a strategy for cancer treatment based on an Shp2 inhibitor.

Major concerns

1. Regarding the Microarray and Bioinformatics analysis it should be clearly indicated the methodology used to obtain the false discovery rate (FDR) and how was the significance-level adjustment performed. FDR values (and not only Fc values) should be indicated in the Tables.

2. In the case of Shp2 ablation of PyMT mammary gland tumours (FIG. 7) it is not mentioned the number of mice used. Also, the levels of Ki67, EYFP, CK8, SAβG and caspase-3 positive cells must be quantified at different stages of tumour development.

3. Although SAβG activity is observed in EYFP-positive areas, but not in EYFP-negative ones (FIG. 7B) it is not shown whether these cells are positive for p53 or p27.

4. A key issue to support the proposed mechanism is to validate in vivo the upregulation of Skp2, Aurka and DII1/Hey1 genes by Shp2.

5. The authors show that treatment of PyMT;coShp2 mice with GS493 blocks tumour formation. However, it is essential to demonstrate that this inhibitor induces cellular senescence in vivo (SAβG activity, exclusion of Ki67, senescence-associated heterochromatin markers, and the expression of p53 or p27). Moreover, PyMT;Shp2fl/fl and PyMT;MMTV-cre;Shp2+/fl animals should be included in this study.

Minor points

6. Also, in the Microarray and Bioinformatics analysis, it is usually recommended to apply Limma Package (Bioconductor) instead of a Student's t-test to check differential expression of genes.

7. For non-specialists, it should be indicated in the text that CK8 is used to as a marker of mammary normal and cancer cells

8. It is not clarified how Skp2 and Aurora A may regulate p27 or p53, or whether DII1/Hey1 controls senescence by regulating p53, p27 or both mediators of senescence.

9. In my opinion, the proposed mechanism is too ambitious. Chemical inhibitors for Src (by PP2), Fak (by TAE226), Mek1 (by U0126), NAE (by MLN4924), Aurora A (by VX-680) and Notch (by DAPT) were not used in an in vivo context.

Referee #2:

Lan et al. investigated the role of the protein-tyrosine phosphatase, Shp2, in breast cancer. They found that genetic loss of Shp2 and inhibition of Shp2 induced senescence in PyMT tumor cells to a similar extent. Expression profiling indicated that particularly genes that are involved in the cell cycle, DNA replication and p53 signaling were downregulated in Shp2 knockout and Shp2 inhibitor-treated PyMT spheres. Expression of selected target genes partially rescued senescence. Shp2 inhibition reduced Src, Fak and Mek1/Erk signaling and inhibition of Src, Fak and Mek1 inhibited expression of selected Shp2 target genes. Re-analysis of public databases indicated a correlation between Shp2-regulated gene expression and survival of breast cancer patients. Shp2 knockout or inhibition suppressed PyMT tumor formation in mice. Finally, Shp2 knockout induced senescence in PyMT in mice. The data led the authors to propose a model for Shp2 signaling via kinases and target gene expression tot control senescence. The authors conclude that therapies which rely on senescence induction by inhibiting Shp2 or controlling target gene products may be useful in blocking breast cancer.

This is an interesting paper that reveals a novel role for Shp2 in breast cancer with potentially important implications for treatment of breast cancer patients.

Major points:
1. Shp2 knockout or inhibition led to downregulation of many genes (Table S1). Four genes were selected for further analysis (Fig. 2). What was the rationale to select these four genes? Expression of two of these genes, Skp2 and Aurka, led to a partial rescue of Shp2-induced senescence (Fig. 2C-E). Would combined expression of Skp2 and Aurka enhance the rescue? N3IC fully rescued Shp2 knockout-induced senescence, which might suggest that the effect is mediated solely by Notch signaling. This should be explained.
2. Fig. 4. There is a correlation between Shp2-regulated gene expression and survival of breast cancer patients, particularly with some of the gene classes that are most strongly regulated. Fig. S4 shows that there is no correlation between all up- or downregulated genes and survival. This is puzzling. Given the positive correlation with some gene classes (Fig. 4), one would expect an inverse correlation with other gene classes in order for all genes not to have a correlation. Was this observed? How can one explain such an inverse correlation?
3. Fig. 7C. The model presented here is clear and helpful. However, no data are provided for some of the arrows. Particularly the inhibitory arrows from Skp2,Aurka and Dll1/Hey1 to p27,p53 need to be clarified experimentally (Discussion, p.14). Question marks should be added next to these arrows to clarify the status of these arrows.

Referee #3:

This manuscript describes the results of a study of the consequences of ablation of SHP2 in a commonly used murine model of mammary carcinogenesis. SHP2, encoded by the PTPN11 gene, is a ubiquitously expressed tyrosine phosphatase that transduces mitogenic, pro-survival, cell-fate and/or pro-migratory signals from numerous growth factor, cytokine, and extracellular-matrix receptors. The model employs the PyMT oncogene that, although not a factor itself in human cancers, is known to activate several conserved oncogenic pathways. Using a state-of-the-art inducible transgenic method for SHP2 ablation, the investigators find that acute knockout of SHP2 leads to senescence of the incipient tumor cells. Not surprisingly, there is a high degree of selection for tumor cells that have lost the ability to excise the floxed SHP2 gene, and the investigators show that these cells ultimately take over cultures and produce tumors.

An interesting point that deserves more discussion is the finding that a small molecule SHP2 inhibitor, GS493, developed in the senior investigator's laboratory, can apparently suppress the growth of these surviving tumor cells when used at doses that are well tolerated by the host animals. Given the essential role that SHP2 has been shown to play in development and maintenance of various organs, it is pleasantly surprising that GS493 appears to inhibit the growth of tumor cells without causing adverse effects in normal cells. The authors should address the possible mechanistic reasons for this difference in susceptibility and the potential for a favorable therapeutic index.

In their efforts to understand the nature of the senescence response to SHP2 ablation, the investigators performed transcriptional profiling experiments. Unsurprisingly, since the comparisons were made between actively dividing and non-dividing cells, many genes involved in cell cycle

progression and DNA replication were differentially expressed. It is not clear why the investigators chose to focus on the particular genes Skp2, Aurka, and Dll1. More rationale needs to be provided.

The title of the paper indicates that "SHP2 signaling is essential to the suppression of senescence." However, using transduced cDNAs or specific inhibitors, the investigators showed that altering the activity of several different pathways was sufficient to avoid senescence of the PyMT-expressing cells in which SHP2 was knocked out. A pseudo-linear series of events is proposed to result in the senescence response. However, experimental support for this scheme is limited; no direct link between SHP2 and the deregulated genes was established. Did the investigators find any growth inhibitory regimens that did not result in senescence? The alternative possibility that it is general imbalances in signals emanating from growth promoting pathways, rather than suppression of a specific SHP2-generated signal, that induce the senescent response, is not considered. The distinction between these possible explanations is important, as it will inform further efforts to design pro-senescence regimens. PyMT is a particularly potent oncogene that may make cells particularly sensitive to senescence by multiple regimens. However, tumors lacking such potent oncogenes, or which retain the capacity to modulate oncogene expression, may be more tolerant of SHP2 inhibition - this possibility needs to be addressed or at least discussed. A more accurate and circumspect title would specifically mention PyMT.

It is curious that the survival analyses did not show that overall expression of all SHP2-deregulated genes was predictive of patient outcome. Moreover, SHP2 expression was a less significant predictor than cell cycle or DNA replication genes. Is SHP2 expression in human breast cancer patients merely a reflection of the percentage of dividing cells or do the authors have reason to consider it an independent predictor?

Minor concerns:
- The first paragraph of the Introduction is overly broad with limited relevance to the work described. It could be omitted.
- In the third paragraph, it is not clear what the authors mean by the statement that "senescence is essential in human and mouse tumors...." Also, the statement that "senescence may also be used in pro-senescence therapies" does not make sense.

1st Revision - authors' response 12 September 2014

General response to all reviewers:
We would like to thank the reviewers for their critical and insightful comments to our manuscript. We have now made a major revision of the manuscript and addressed the concerns raised. The microarray and bioinformatics analyses are now described in more detail, and the rationale for choosing to focus on *Skp2*, *Aurka*, *Dll1* and *Hey1* is explained further. By experiments, we have clarified the links between Shp2 target genes (*Skp2*, *Aurka*, *Dll1* and *Hey1*) and the downstream senescence effectors (p27 and p53). Moreover, we have validated the regulation of the target genes and senescence effectors by Shp2 in *in vivo* contexts.

Response to Referee #1:
In this manuscript entitled "Shp2 signaling is essential to the suppression of senescence in mammary gland cancer in mice" the authors provide evidence that the genetic ablation or pharmacological blockage of Shp2 inhibits cell proliferation and the self-renewal capacity of breast PyMT tumour cells. The Shp2 ablation and inhibition correlates with the induction of cellular senescence, and Shp2-regulated gene expression programs predict the survival of breast cancer patients. In addition, they propose a strategy for cancer treatment based on an Shp2 inhibitor.
Major concerns
1. Regarding the Microarray and Bioinformatics analysis it should be clearly indicated the methodology used to obtain the false discovery rate (FDR) and how was the significance-level adjustment performed. FDR values (and not only Fc values) should be indicated in the Tables.

Answer: We have now provided a more precise description about the methodology used to obtain FDR and the adjustment of the significance levels in the Materials and Methods, page 21, lines 14-18. We have also added FDR values in the Tables.

2. In the case of Shp2 ablation of PyMT mammary gland tumours (FIG. 7) it is not mentioned the number of mice used. Also, the levels of Ki67, EYFP, CK8, SAβG and caspase-3 positive cells must be quantified at different stages of tumour development.
Answer: We now specified the number of mice used in the new Fig. 8, in the figure legend. We also quantified the numbers of cells positive for Ki67, EYFP, CK8 and SA-β-gal (SAβG) at hyperplasia, adenoma and carcinoma stages of tumor development: the results showed a gradual loss of EYFP+ recombined cells and SAβG+ senescent cells and a steady gain of Ki67+ proliferating cells during tumor development, while the number of cells expressing the pan-epithelial marker Keratin 8 (CK8) remained stable (new Supplementary Fig. S6). Since cleaved caspase-3 remained undetectable at all stages (new Supplementary Fig. S6A), we did not quantify it.

3. Although SAβG activity is observed in EYFP-positive areas, but not in EYFP-negative ones (FIG. 7B) it is not shown whether these cells are positive for p53 or p27.
Answer: This is an important point. We now performed Western blot analyses on EYFP+ and EYFP- tumor cells freshly isolated from endogenous tumors from PyMT;MMTV-Cre;Shp2$^{fl/fl}$;EYFP$^{-/fl}$ mice: the results show that both p53 and p27 proteins were indeed significantly increased in EYFP+ cells that had lost Shp2 (new Supplementary Fig. S8C).

4. A key issue to support the proposed mechanism is to validate in vivo the upregulation of Skp2, Aurka and DII1/Hey1 genes by Shp2.
Answer: This is a critical point. We now performed qRT-PCR analyses on EYFP+ and EYFP- tumor cells freshly isolated from endogenous tumors from PyMT;MMTV-Cre;$^{Shp2fl/fl}$;EYFP$^{-/fl}$ mice: the result show that all genes Skp2, Aurka, Dll1 and Hey1 were downregulated in EYFP+ cells that had lost Shp2 (new Supplementary Fig. S8B). The data thus confirm that these genes are under the regulation of Shp2 in vivo.

5. The authors show that treatment of PyMT;coShp2 mice with GS493 blocks tumour formation. However, it is essential to demonstrate that this inhibitor induces cellular senescence in vivo (SAβG activity, exclusion of Ki67, senescence-associated heterochromatin markers, and the expression of p53 or p27). Moreover, PyMT;Shp2fl/fl and PyMT;MMTV-cre;Shp2+/fl animals should be included in this study.

Answer: We agree that it would be informative to demonstrate the senescence induction effect of GS493 in vivo and to include PyMT;Shp2$^{fl/fl}$ and PyMT;MMTV-Cre;Shp2$^{+/fl}$ animals in this study. We first need to mention that PyMT;coShp2 mice develop no tumors; so, senescence is not easy measurable. Moreover, since we provided solid evidence that Shp2-recombined tumor cells underwent senescence in PyMT;coShp2 mice, and that GS493 potently induced senescence in vitro, we reason that the tumor suppressive effect of GS493 in PyMT;coShp2 mice must indeed be due to its function in senescence induction. Furthermore, to perform these mouse experiments might require half a year or longer: we therefore believe that they should be considered outside of the scope of the present manuscript.

Minor points

6. Also, in the Microarray and Bioinformatics analysis, it is usually recommended to apply Limma Package (Bioconductor) instead of a Student's t-test to check differential expression of genes.

Answer: We have described the methodology more precisely in the Materials and Methods, page 21, lines 14-18. Specifically, the analysis was performed in the R statistical environment, and the ANOVA test followed by FDR adjustment was applied to identify differential expression of genes.

7. For non-specialists, it should be indicated in the text that CK8 is used to as a marker of mammary normal and cancer cells.

Answer: We now used CK8 as a marker of normal mammary gland epithelial and tumor cells in the text as well as in the figure legends.

8. It is not clarified how Skp2 and Aurora A may regulate p27 or p53, or whether DII1/Hey1 controls senescence by regulating p53, p27 or both mediators of senescence.

Answer: We have now performed a series of additional critical experiments to clarify the link between the genes Skp2, Aurora A and Dll1/Notch and the senescence effectors p27 and p53, including examination of p27 and p53 protein levels in various cells (cDNA-rescued Shp2 ko cells and inhibitor-treated cells) and rescue assays on inhibitor-treated cells with knockdown of p27 or p53. The overall data demonstrate that p27 indeed acts downstream of Skp2, and that p53 acts downstream of Aurora A and Dll1/Notch3 to control senescence (new Fig. 3D-H). The biochemical mechanism of how Skp2 regulates p27 and how Aurora A and Notch control p53 needs further investigation, as we say in the Discussion, page 16, lines 1-3.

9. In my opinion, the proposed mechanism is too ambitious. Chemical inhibitors for Src (by PP2), Fak (by TAE226), Mek1 (by U0126), NAE (by MLN4924), Aurora A (by VX-680) and Notch (by DAPT) were not used in an in vivo context.

Answer: Based on our new data (Fig. 3), we believe that the proposed mechanistic model is now much clearer, although ambitious as we agree. We also believe that the use of all these chemical inhibitors in an in vivo context would exceed the scope of the present paper, and would also be very expensive.

Response to Referee #2:

Lan et al. investigated the role of the protein-tyrosine phosphatase, Shp2, in breast cancer. They found that genetic loss of Shp2 and inhibition of Shp2 induced senescence in PyMT tumor cells to a similar extent. Expression profiling indicated that particularly genes that are involved in the cell cycle, DNA replication and p53 signaling were downregulated in Shp2 knockout and Shp2 inhibitor-treated PyMT spheres. Expression of selected target genes partially rescued senescence. Shp2 inhibition reduced Src, Fak and Mek1/Erk signaling and inhibition of Src, Fak and Mek1 inhibited expression of selected Shp2 target genes. Re-analysis of public databases indicated a correlation between Shp2-regulated gene expression and survival of breast cancer patients. Shp2 knockout or inhibition suppressed PyMT tumor formation in mice. Finally, Shp2 knockout induced senescence in PyMT in mice. The data led the authors to propose a model for Shp2 signaling via kinases and target gene expression to control senescence. The authors conclude that therapies which rely on senescence induction by inhibiting Shp2 or controlling target gene products may be useful in blocking breast cancer.

This is an interesting paper that reveals a novel role for Shp2 in breast cancer with potentially important implications for treatment of breast cancer patients.

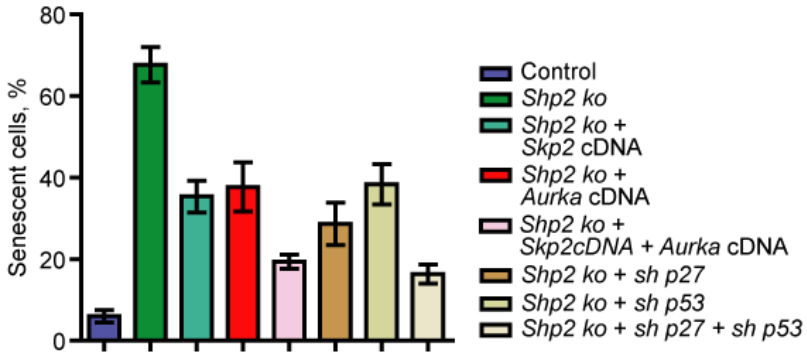
Answer: We are grateful for the reviewer's appreciation of our paper.

Major points:

1. Shp2 knockout or inhibition led to downregulation of many genes (Table S1). Four genes were selected for further analysis (Fig. 2). What was the rationale to select these four genes? Expression of two of these genes, Skp2 and Aurka, led to a partial rescue of Shp2-induced senescence (Fig. 2C-E). Would combined expression of Skp2 and Aurka enhance the rescue? N3IC fully rescued Shp2 knockout-induced senescence, which might suggest that the effect is mediated solely by Notch signaling. This should be explained.

Answer: We now explained our reasoning for the selection of target genes in more detail in Results and Discussion, page 7, lines 25-31, and page 14 and 15, lines 29-33 and 1-14, respectively. We have chosen these genes based on the facts 1) that these genes are deregulated in human breast cancers, such as upregulation of Skp2, amplification of Aurka and translocation of Notch, 2) that Skp2 and Notch3 are prognostic factors for the outcome of human breast cancer patients, and 3) that Skp2, Aurka and Notch have been implicated in senescence regulation in some other cancers.

We also found that combined expression of Skp2 and Aurka enhanced the rescue in an additive manner (see the purple column in the figure attached below). Full rescue by *N3IC may not mean that Notch signaling solely mediates Shp2 function, since Notch inhibition only partially phenocopied Shp2 deficiency (see Supplementary Fig. S2). N3IC is a constitutively active form of Notch3, and constitutive activation of Notch may lead to an excess of signaling. We have discussed this issue on page 15, lines 14-18.*



2. Fig. 4. There is a correlation between Shp2-regulated gene expression and survival of breast cancer patients, particularly with some of the gene classes that are most strongly regulated. Fig. S4 shows that there is no correlation between all up- or downregulated genes and survival. This is puzzling. Given the positive correlation with some gene classes (Fig. 4), one would expect an inverse correlation with other gene classes in order for all genes not to have a correlation. Was this observed? How can one explain such an inverse correlation?

Answer: These are interesting points. We computed the correlation to survival for each gene in the down- or up-regulated list and observed that each list indeed contained some genes associated with worse survival and some others associated with better survival. This may not be surprising because of the complexity of senescence, which is controlled by Shp2 in PyMT tumor cells. Senescence is a collective phenotype, including cell-cycle arrest, chromatin remodeling, deregulation of metabolism and induced secretome (also called senescence-associated secretory phenotype, SASP) (Salama et al, 2014). The functions of some aspects of senescence such as SASP in human cancers remain unknown or are still controversial (Coppe et al, 2010). For instance, we observed a number of inflammatory cytokines associated with SASP in the upregulation gene list. Unlike p53 target genes in the list, these cytokines can be tumor-promoting and correlate with worse prognosis, though they

are negatively regulated by Shp2. In this scenario, a detailed stratification of the Shp2-regulated genes might be more precise. We have not added these data to the paper.

3. Fig. 7C. The model presented here is clear and helpful. However, no data are provided for some of the arrows. Particularly the inhibitory arrows from Skp2, Aurka and Dll1/Hey1 to p27, p53 need to be clarified experimentally (Discussion, p.14). Question marks should be added next to these arrows to clarify the status of these arrows.

Answer: We are pleased that this reviewer appreciated our model. These are indeed critical points. We have now performed a series of additional critical experiments to clarify the link between the genes Skp2, Aurora A and Dll1/Notch and the senescence effectors p27 and p53, including examination of p27 and p53 protein levels in various cells (cDNA-rescued Shp2 ko cells and inhibitor-treated cells) and rescue assays on inhibitor-treated cells with knockdown of p27 or p53. Together, the data demonstrate that p27 act downstream of Skp2, and p53 downstream of Aurora A and Dll1/Notch3 to control senescence (see the new Fig. 3D-H, described on page 8, lines 16-33). The biochemical mechanism of how Skp2 regulates p27 and how Aurora A and Notch control p53 needs further investigation. This is discussed on page 16, lines 1-3.

Response to Referee #3:

This manuscript describes the results of a study of the consequences of ablation of SHP2 in a commonly used murine model of mammary carcinogenesis. SHP2, encoded by the PTPN11 gene, is a ubiquitously expressed tyrosine phosphatase that transduces mitogenic, pro-survival, cell-fate and/or pro-migratory signals from numerous growth factor, cytokine, and extracellular-matrix receptors. The model employs the PyMT oncogene that, although not a factor itself in human cancers, is known to activate several conserved oncogenic pathways. Using a state-of-the-art inducible transgenic method for SHP2 ablation, the investigators find that acute knockout of SHP2 leads to senescence of the incipient tumor cells. Not surprisingly, there is a high degree of selection for tumor cells that have lost the ability to excise the floxed SHP2 gene, and the investigators show that these cells ultimately take over cultures and produce tumors.

An interesting point that deserves more discussion is the finding that a small molecule SHP2 inhibitor, GS493, developed in the senior investigator's laboratory, can apparently suppress the growth of these surviving tumor cells when used at doses that are well tolerated by the host animals. Given the essential role that SHP2 has been shown to play in development and maintenance of various organs, it is pleasantly surprising that GS493 appears to inhibit the growth of tumor cells without causing adverse effects in normal cells. The authors should address the possible mechanistic reasons for this difference in susceptibility and the potential for a favorable therapeutic index.

Answer: We appreciate the reviewer's interest in therapeutic effect of our Shp2 inhibitor. Shp2 indeed plays an essential role in the development and maintenance of many tissues such as the nervous system, kidney and intestine, as our and other laboratories have shown. However, Shp2 is highly upregulated and overactivated in mouse and human breast cancers. For instance, the Shp2 protein is strongly elevated in PyMT tumor cells (see Supplementary Fig. S1), which may be more dependent on high Shp2 activity and thus may be more sensitive to Shp2 inhibition. This appears to provide a therapeutic window for Shp2 inhibitors, in which Shp2 inhibitors remarkably dampen the aberrant activity of Shp2 in tumorous tissues without affecting normal or low levels of activity in normal tissues. We have discussed this in the new version on page 18, lines 4-9. Therefore, establishing the therapeutic window for Shp2 inhibitors is of particular importance.

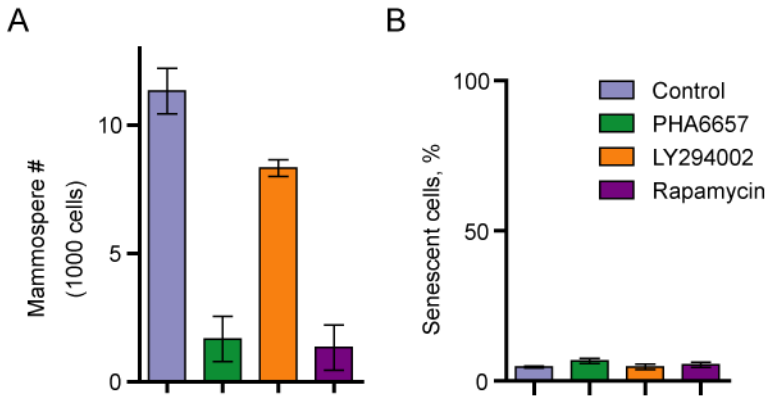
In their efforts to understand the nature of the senescence response to SHP2 ablation, the investigators performed transcriptional profiling experiments. Unsurprisingly, since the comparisons were made between actively dividing and non-dividing cells, many genes involved in cell cycle progression and DNA replication were differentially expressed. It is not clear why the investigators chose to focus on the particular genes Skp2, Aurka, and Dll1. More rationale needs to be provided.

Answer: We have now explained our reasoning in more detail in the Results and Discussion of the revised manuscript. We chose these genes based on the facts 1) that these genes are deregulated in human breast cancers, such as upregulation of Skp2, amplification of Aurka and translocation of Notch, 2) that Skp2 and Notch3 are prognostic factors for outcomes of human breast cancer patients, and 3) that Skp2, Aurka and Notch have been implicated in senescence regulation in some other cancers. This reasoning is now described on page 7, lines 25-31, and page 14 and 15, lines 29-33 and 1-14, respectively.

The title of the paper indicates that "SHP2 signaling is essential to the suppression of senescence." However, using transduced cDNAs or specific inhibitors, the investigators showed that altering the activity of several different pathways was sufficient to avoid senescence of the PyMT-expressing cells in which SHP2 was knocked out. A pseudo-linear series of events is proposed to result in the senescence response. However, experimental support for this scheme is limited; no direct link between SHP2 and the deregulated genes was established. Did the investigators find any growth inhibitory regimens that did not result in senescence? The alternative possibility that it is general imbalances in signals emanating from growth promoting pathways, rather than suppression of a specific SHP2-generated signal, that induce the senescent response, is not considered. The distinction between these possible explanations is important, as it will inform further efforts to design pro-senescence regimens. PyMT is a particularly potent oncogene that may make cells particularly sensitive to senescence by multiple regimens. However, tumors lacking such potent oncogenes, or which retain the capacity to modulate oncogene expression, may be more tolerant of SHP2 inhibition - this possibility needs to be addressed or at least discussed. A more accurate and circumspect title would specifically mention PyMT.

Answer: We have now strengthened our mechanistic model by carrying out additional experiments, including examination of p27 and p53 protein levels in various cells (cDNA-rescued Shp2 ko cells and inhibitor-treated cells) and rescue assays on inhibitor-treated cells with knockdown of p27 or p53. The data clearly established a link between Shp2 target genes and senescence effectors.

We excluded the possibility that the senescence response may be the result from general imbalances in growth-promoting signals, since we found that inhibitors against Met (PHA6657), Pi3k (LY294002) or mTOR (Rapamycin) inhibited proliferation but did not induce senescence (see the figure below). This is now mentioned on page 16, lines 30-32.



Although the senescence suppression function of Shp2 was identified in PyMT tumors, it may not be limited to the PyMT oncogene context, since PyMT mimics many potent human oncogenes such as Ras, Src and multiple RTKs. In addition, inhibition of the products of Shp2 target genes Skp2 and Aurka has already been shown to induce senescence in several types of human cancers (Huck et al, 2010; Lin et al, 2010; Paget et al, 2012). We envision that the senescence-suppressive function of Shp2 may be important for human cancers that are addicted to strong oncogenes. Since we have also identified the Shp2 gene signatures that have prognostic values in clinical settings, we believe that the title of the paper without mentioning PyMT is acceptable.

It is curious that the survival analyses did not show that overall expression of all SHP2-deregulated genes was predictive of patient outcome. Moreover, SHP2 expression was a less significant predictor than cell cycle or DNA replication genes. Is SHP2 expression in human breast cancer patients merely a reflection of the percentage of dividing cells or do the authors have reason to consider it an independent predictor?

Answer: The lack of correlation of overall expression of all Shp2-regualted genes and the less significant correlation of Shp2 with patient outcome could be due to the complexity of senescence, which is a collective phenotype including cell-cycle arrest, chromatin remodeling, deregulation of metabolism, and induction of secretome (also called senescence-associated secretory phenotype, SASP). The functions of some aspects of senescence such as SASP in human cancers remain unknown or are still controversial (Coppe et al, 2010). For instance, we observed a number of inflammatory cytokines associated with SASP in the upregulation gene list. These cytokines may be tumor-promoting and correlate with worse prognosis, though they are negatively regulated by Shp2. In this scenario, a detailed stratification of all Shp2-regulated genes would be more precise. We have planned such experiments.

Shp2 regulates many aspects of tumor biology in human breast cancer, like cancer stem cell stemness, epithelial-mesenchymal transiton (EMT), proliferation and migration, and therefore it is not merely a marker for dividing cells. Based on our analysis and recent publications (Muenst et al, 2013; Sausgruber et al, 2014), Shp2 is indeed an independent predictor for human breast cancer.

Minor concerns:

- The first paragraph of the Introduction is overly broad with limited relevance to the work described. It could be omitted.

Answer: We agree that this paragraph is broad, and we have therefore shortened it. We believe that the revised form will now better prepare readers.

- In the third paragraph, it is not clear what the authors mean by the statement that "senescence is essential in human and mouse tumors...." Also, the statement that "senescence may also be used in pro-senescence therapies" does not make sense.

Answer: We changed the first sentence into "Senescence occurs during development of human and mouse tumors". We changed the second sentence into "Inducing senescence by therapeutic regimens may be used as a strategy for cancer treatment".

References

Coppe JP, Desprez PY, Krtolica A, Campisi J (2010) The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annu Rev Pathol* **5:** 99-118

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2nd Editorial Decision 23 September 2014

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been re-reviewed by the three referees.

As you can see below, the referees appreciate the introduced changes and are overall very supportive of publication here. However, referee #1 also requests one additional experiment before publication here - namely to treat the mice with the Shp inhibitor after PyMT tumor induction and then validate your findings in this model. This was an issue that was raised originally as well, but I also see that there might have been some confusion regarding this point. However this experiment shouldn't be too extensive to take on and I would appreciate if you would undertake such an experiment, as it would strengthen the findings reported. This is the last issue to sort out.

REFEREE REPORTS

Referee #1:

The authors have improved the paper and have addressed some of my comments. However, I must say I expected them to address the point that I considered "essential" (my point #5), that is, to generate PyMT tumors, treat the mice with the SHP2i, and examine the tumors for arrest/regression, signs of senescence (SAbG), and in vivo validation of their model (upregulation of Skp2, p27, Aurka, p53, etc). This should not take more than one month and this would certainly improve the quality of the paper. In other words, the same experiment they show in Fig. 6D, but instead of treating from week 7 to week 10, to treat the mice after tumor formation (days 30 and beyond according to Fig. 6E). As it is, their model is validated in pre-tumoral PyMT mammary gland, but not in established PyMT tumors in vivo. This is not out of the scope of the present manuscript and it does not entail months and months of work.

Minor points

1. Figure S8 should be moved to the main text and combined with current Figure 8.
2. Although the authors show increased protein levels of p53 and p27 in EYFP+ cells it would be definitive if they can show that the expression of these senescence effectors in PyMT;MMTV-Cre;coShp2 tumours colocalizes with the SAβG-stained areas.
3. Also, is there any correlation between senescence and p16 and/or p19ARF in these tumours? these are major mediators of senescence in many experimental systems.

Referee #2:

The concerns I raised on the original manuscript were addressed satisfactorily in the revised version.

Referee #3:

The manuscript is now suitable for publication. However, a more accurate and circumspect title would specifically mention PyMT. In addition, the introduction still contains information that is not directly relevant to the study, and therefore could be further shortened without compromising readers' ability to interpret the study.

2nd Revision - authors' response 20 January 2015

Response to Referee #1:

The authors have improved the paper and have addressed some of my comments. However, I must say I expected them to address the point that I considered "essential" (my point #5), that is, to generate PyMT tumors, treat the mice with the SHP2i, and examine the tumors for arrest/regression, signs of senescence (SAbG), and in vivo validation of their model (upregulation of Skp2, p27, Aurka, p53, etc). This should not take more than one month and this would certainly improve the quality of the paper. In other words, the same experiment they show in Fig. 6D, but instead of treating from week 7 to week 10, to treat the mice after tumor formation (days 30 and beyond according to Fig. 6E). As it is, their model is validated in pre-tumoral PyMT mammary gland, but not in established PyMT tumors in vivo. This is not out of the scope of the present manuscript and it does not entail months and months of work.

Answer: We now performed a therapy experiment by treating already tumor-bearing PyMT mice with the Shp2 inhibitor GS493, followed by analyzing the kinetics of tumor growth, senescence and its associated effectors, and Shp2 target genes in these tumors. The results show that GS493 remarkably inhibits the growth of the preformed PyMT tumors, particularly at late phases of treatment (3 weeks of treatment and later on), where tumor growth is fully halted (new Fig. 8E). The results also show that GS493 strongly induces senescence, activates senescence effectors p27 and p53, and downregulates Shp2 target genes (new Supplementary Fig. 8C-E). The data thus provide solid evidence that Shp2 inhibition by GS493 impedes tumor growth by inducing senescence, also suggesting the therapeutic value of Shp2 inhibitors in human breast cancer. Note that PyMT instead of PyMT;coShp2 mice were used in this experiment: tumors are identical in both genotypes once tumors are palpable.

Minor points

1. Figure S8 should be moved to the main text and combined with current Figure 8.

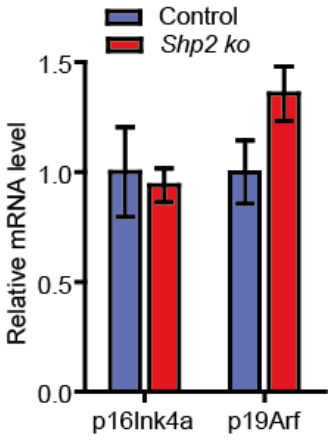
We now merged Figure S8 into Figure 8 to create the new Fig. 8.

2. Although the authors show increased protein levels of p53 and p27 in EYFP+ cells it would be definitive if they can show that the expression of these senescence effectors in PyMT;MMTV-Cre;coShp2 tumours colocalizes with the SAβG-stained areas.

We now performed immunofluorescence staining of EYFP, p53, and p27 in tumor sections from control and PyMT;coShp2 mice carrying the EYFP allele. The results clearly show co-localization of p53 and p27 with EYFP in tumors from PyMT;coShp2 mice (new Fig. 8B). Since EYFP also co-localizes with SA-β-gal staining (new Fig. 8A), it is clear that p53 and p27 co-localize with the SA-β-gal areas.

3. Also, is there any correlation between senescence and p16 and/or p19ARF in these tumours? these are major mediators of senescence in many experimental systems.

p16Ink4a and p19Arf are indeed very important effectors of senescence in many experimental models. In PyMT tumors in the absence of Shp2, however, the expression of p16Ink4a and p19Arf seems not significantly induced: 1) both genes are absent in the deregulated gene lists from our microarray data; 2) qRT-PCR analysis confirmed that p16Ink4a is not induced and p19Arf is slightly upregulated in Shp2 ko senescent cells (see the figure below).



Response to Referee #2:

The concerns I raised on the original manuscript were addressed satisfactorily in the revised version.

We are happy that the referee was satisfied with our revision.

Response to Referee #3:

The manuscript is now suitable for publication. However, a more accurate and circumspect title would specifically mention PyMT. In addition, the introduction still contains information that is not directly relevant to the study, and therefore could be further shortened without compromising readers' ability to interpret the study.

We now mention PyMT in the new title. We also further shortened and modified the first paragraph of the Introduction. We believe that the revised form will now well prepare readers.

Accepted 04 February 2015

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been re-reviewed by referee #1 who appreciate that the added data. I am therefore very pleased to accept the paper for publication here.

Congratulations on a very nice paper

REFEREE REPORT

Referee #1:

The reviewers have addressed my comments. I congratulate the authors for the high quality and relevance of their work.

