

Manuscript EMBO-2016-93889

FBW7 suppression leads to SOX9 stabilization and increased malignancy in medulloblastoma

Aldwin Suryo Rahmanto, Vasil Savov, Andr  Brunner, Sara Bolin, Holger Weishaupt, Alena Malyukova, Gabriela Ros n, Matko  an er, Sonja Hutter, Anders Sundstr m, Daisuke Kawauchi, David T. W. Jones, Charles Spruck, Michael D. Taylor, Yoon-Jae Cho, Stefan M. Pfister, Marcel Kool, Andrey Korshunov, Fredrik J. Swartling and Olle Sangfelt

Corresponding authors: Fredrik J. Swartling, Uppsala University and Olle Sangfelt, Karolinska Institutet

Review timeline:

Submission date:	16 January 2016
Editorial Decision:	26 February 2016
Revision received:	05 July 2016
Editorial Decision:	02 August 2016
Revision received:	12 August 2016
Accepted:	18 August 2016

Editor: Hartmut Vodermaier

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

26 February 2016

Thank you again for submitting your manuscript to The EMBO Journal. We have now received feedback from three expert referees, whose reports are copied below for your information. These referees consider the topic of your study as well as its results potentially interesting and important, yet raise a number of substantial concerns that would need to be satisfactorily addressed before publication might be warranted. Since these issues are well-taken and should in principle be addressable through a regular (major) revision of the study, I would like to invite you to prepare a revised manuscript in response to the reviewers' comments.

As our editorial policies allow for only a single round of major revisions, I should point out that it will be essential to carefully respond to all raised points during this round. Particular attention should be given to the concerns of referees 1 and 3 related to the *in vivo* data, their statistical significance and possible bias inherent in them. Another important point relates to referee 1's issues with the mechanistic experiments in Figures 1 and 2, which would need to be strengthened through additional work. Moreover, the overall impact of the manuscript for the field would clearly be increased by extending current *in vitro* experiments on Fbw7/Sox9 roles in resistance to therapy to *in vivo* settings, and providing some more insight into the underlying mechanisms (see referees 1 and 2). I will not repeat the remaining points in detail here, since they are well-explained in the referee reports, but would like to encourage you to get back to me with any specific questions/comments arising from the reports. Finally, please take note of the EMBO Journal article

format guidelines, in particular please make sure to include a sufficiently informative Material and Methods section in the main manuscript.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively affect our final decision on 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 COMMENTS

Referee #1:

This paper from the Sangfeldt lab shows that the Sox9 transcription factor is degraded by the SCF-Fbxw7 ubiquitin ligase, and describes a role for these interactions in the pathogenesis, and possibly the treatment of, medulloblastomas associated with SHH pathway activation. The identification of Sox9 as an Fbxw7 substrate initially fell out of a proteomic screen. While I have some specific comments below, in general the data support the idea that Sox9 can behave as a canonical Fbxw7 substrate in which Sox9 degradation is dependent upon its consensus degron and phosphorylation by GSK-3.

The paper next moves on to the physiologic role of Fbxw7-mediated degradation in medulloblastoma pathogenesis, migration and invasion. The choice of medulloblastoma is quite interesting, in light of the common Fbxw7 mutations found in this disease, and the role of Sox9 in this disease. While the data support the conclusions in this specific system, because these models rely on Sox9 and/or Fbxw7 overexpression, I feel they fall short of demonstrating a true physiologic role for Sox9 stabilization by Fbxw7 mutations in naturally occurring medulloblastomas. Finally, because AKT and GSK3 are antagonistic, the paper examines the role of AKT inhibitors in therapy. This has always seemed like logical approach for Fbxw7-associated cancers, given the near-universal role of GSK3 in instigating substrate degradation, but has typically not been successful. These data look more promising, at least with respect to Sox9 abundance. However, the mechanisms underlying the synthetic lethality between reduced Sox9 abundance and cisplatin is totally mysterious, and the specific role of Sox9 degradation in mediating the effect of the AKT inhibitor is shown in only a single overexpression experiment in supplemental Fig. 5.

Overall I suspect that the identification of Sox9 as a bona fide Fbxw7 substrate is correct, but the physiologic significance of this pathway in medulloblastoma pathogenesis is less convincing.

Specific Points.

Fig. 1. The data are clear that Sox9 and Fbxw7 interact, and that GSK3 phosphorylates Sox9 on T236. Panel F is a little confusing, because efficient Fbxw7 binding usually requires both CPD phosphorylation, and GSK3 does not appear to phosphorylate T240. What is the affinity of this interaction? Is T240 phosphorylated? If not, is the binding independent of T240? Finally, is the GSK3 T326 phosphorylation primed by T240 phosphorylation? This appears not to be the case, and has important implications for understanding the impact of the AKT inhibitors, which has differential effects on primed versus non-primed GSK3 substrates.

Fig. 2. These data nicely show that endogenous Fbxw7 regulates endogenous SOX9 stability in 293 cells, which I presume is the cell type in which the original proteomics were performed?
2D. Why is the abundance of Fbxw7 so different in the various con-transfections? It is hard to determine a specific lack of degradation of the phosphorylation site mutants when Fbxw7 abundance seems much lower in these lanes.

2G. This experiment is flawed because the IP-western is done in the direction that would simply detect any ubiquitylated protein bound to Sox (including Fbxw7 itself), as opposed to showing Sox9-ubiquitin conjugates. The IP should be done in the other direction to show specific Sox9 immunoreactive conjugates, and also with mutant Fbxw7, as another control.

2H. The background in this in vitro experiment seems quite high (in the lane without Sox9). An important control, the use of mutant Fbxw7, is also missing.

Fig 3. This figure, which shows that Sox9 is an Fbxw7 substrate in medulloblastoma cells, is much less convincing than the 293 cell experiments.

2B. The difference in these survival curves may be significant, but is very modest and these data could reflect any Fbxw7 substrate(s) other than Sox9.

3D. These overexpression assays are not sufficient to show that Fbxw7 degrades Sox9 in medulloblastoma cells. The authors should consider using the same knockdown approach to study endogenous proteins they used in 293 cells

Fig. 4. Although the survival curve advantage in 4A is modest, I do think this figure overall supports the idea that Fbxw7 modulates pathogenesis and survival in these two Sox9-overexpression driven systems. However, if one accepts the idea that overexpression Fbxw7 degrades overexpressed Sox9 in medulloblastoma cells, I find these approaches somewhat contrived, since it seems almost certain that these results would be obtained. The data do not show a role for Sox9 stabilization in a medulloblastoma with a natural Fbxw7 mutation.

Fig. 6. These data do show a modest sensitization platinum sensitivity cause by Sox9 overexpression, and an even more reversal of this toxicity by Fbxw7 overexpression. Again the system seems set up just show just this affect.

What are the supposed mechanisms through which Sox9 abundance mediates the synthetic lethality between platinum and AKT inhibition?

6D. Nice! This pretty clearly shows AKT-modulation of Fbxw7-driven Sox9 turnover.

6F. These data do not show that Sox9 stability is involved with the synthetic lethality. The only data that speak to this are in EV5D, but the amount of Sox9 overexpression isn't shown. Do normal amounts of Sox 9 expression (that is resistant to Fbxw7) also rescue the synthetic lethality?

Referee #2:

SOX9 is a transcription factor expressed in the majority of solid tumors where it plays various roles depending on tumor type such as regulating cell invasion, differentiation and survival. However, little is known about how post-translational modifications regulate SOX9 function.

In this manuscript, Rahmanto et al. demonstrate that SOX9 interacts with FBW7, an ubiquitin ligase. Using biochemical experiments in vitro, the authors showed that FBW7 recognizes phosphorylated SOX9 by GSK3b at a conserved residue. This interaction leads to ubiquitination and degradation of SOX9. Using mutagenesis, they show that mutations of this phosphorylation site leads to Sox9 stabilization.

Later, Rahmanto and colleagues uses gene expression profile from primary medulloblastoma and show that FBW7 levels inversely correlate with patient survival of medulloblastoma patients, particularly in the SHH subgroup. Interestingly, whole exome sequencing analysis show that FBW7 is mutated in 11% of SHH medulloblastoma. Using tissue microarray, the authors show that while SOX9 and FBW7 mRNA levels do not correlate, there's an inverse correlation between SOX9 and FBW7 proteins levels within medulloblastoma.

Using inducible FBW7 construct together with SOX9 gain of function in orthotopic transplant of medulloblastoma cell line, the authors show that FBW7 overexpression decreases tumor spreading and migration, resulting in enhanced mice survival. Using Sox9 gain of function in medulloblastoma initiation cells (MIC), they show that SOX9 dramatically increases metastasis and migration of these cells. This increase is reduced by FBW7 overexpression. They also observe a correlation between SOX9 levels and metastasis in medulloblastoma patients and show that SOX9 overexpression promotes the expression of genes involved in EMT.

In addition, they show that overexpression of SOX9 in medulloblastoma cell lines reduces efficiency of cisplatin treatment. Interestingly, this can be counteracted by inhibition of the PI3K/AKT axis which promotes GSK3 activity and therefore SOX9 degradation.

Overall the experiment described in this manuscript are well conducted and controlled. The findings reported brings important insights about the role of Sox9 posttranslational modifications in cancer

progression and response to therapy. While we believe that this manuscript will appeal the readers of EMBO J, we think the manuscript can be further improved by addressing the following points prior to publication:

1. While the observations regarding tumor growth and mice survival are well characterized (Fig 4) it is not clear what's happening at the cellular level. For instance, how are cell death, differentiation, proliferation and invasion affected by perturbation of SOX9, FBW7 or inhibitors treatment? (both in orthotopic transplant and in vitro. The authors should study in more detail these parameters by immunofluorescence.
2. The microarrays analysis are superficially presented. The analysis seems to focus only on few selected genes. The authors should perform Gene ontology analysis and GSEA with previously published data sets analyzing EMT, Sox9 target genes, and any other relevant data sets.
3. The role of FBW7 in regulating cisplatin sensitivity has already been described (Song et al. 2015; Yu et al, 2013). This work must be cited.
4. Regarding the role of Sox9/Fbw7 in the resistance to therapy, all the experiments presented in the manuscript are performed in vitro. It would be nice if the authors can perform these experiments in vivo.
5. While it seems clear that FBW7 low levels/SOX9 high levels are involved in resistance to cisplatin, the mechanism still remains unclear. Could the author provide some experiments/discussion about how SOX9 would promote resistance to therapy in this case?
6. The model presented on Figure 7 seems largely speculative. While the upper part as well as the targeted degradation is well supported by the data, the experimental support for the lower part is much less clear. For instance, the EMT-like reprogramming and stem cell phenotype are poorly supported by the data. This should be removed.

Referee #3:

The authors present data suggesting a role of FBW7 and Sox9 in growth and metastatic spread of medulloblastoma. Whilst FBW7 is downregulated in all subgroups of medulloblastoma, there is a high incidence of FBW7 mutations in the SHH subgroup of medulloblastoma.

A number of experiments demonstrate the role of FBW7 in degrading Sox9, and conversely, a stabilisation of Sox9 by down-regulation or inactivation of FBW7.

The work is logically structured and presents data in a concise and intuitive fashion. First, the authors demonstrate Sox9 interaction with FBW7 through a conserved motif, phosphorylation by GSK3 and experiments demonstrating a Sox9 ubiquitination and degradation independent of GSK3. Comparison of FBW7 expression profiles across databases of primary medulloblastomas shows an inverse correlation of expression with clinical outcome.

These data are further corroborated by testing expression levels of FBW7 and Sox9 mRNA and Sox9 protein on a set of 142 medulloblastomas.

In parallel, xenografting of cells with modulated FBW7 expression shows that down regulation of FBW7 promotes metastatic spread of tumour cells. A borderline significance is seen in the survival.

Generally, the quality of the data is excellent or very high, and the conclusions are supported by the data.

There are a number of minor issues that should be addressed by the authors:

Main issues:

- 1) TMA analysis: clarify how the mRNA levels were quantified? From the materials section, it appears that arbitrary scoring was performed, which is not adequate, in particular if no scoring criteria (number of stained particles, number of nuclei containing particles etc) were defined.

An adequate methodology using digital image quantification on TMA sections should be used. For example, The RNAscope technology enables a fully quantifiable readout using professional image analysis methods, including those offered by the company itself.

Furthermore, the quantification of the Sox9 immunostaining on TMA sections is not specified.

Again, it is essential that this is done using a method to generate quantitative data, which have the benefit of generating continuous and unbiased data. It is further recommended that the authors use an additional Sox9 antibody to corroborate and validate their IHC data. The values/readout of the 2 antibodies should be compared/combined.

Another important rationale for a more advanced image analysis method for the 142 samples is the potential value in correlating the Sox9 levels with the clinical outcome (metastasis and spread).

TMA's are generated from various primary sources which may vary in fixation time, generating a potentially heterogeneous set of TMA cores. Using an antibody derived from a different clone may mitigate some of the problems of TMA. How was the variability of IHC staining accounted for?

Does it explain some of the discrepancies to clinical outcomes as shown in the figure?

Figure 3F: what are the different colours in figure 3F indicate?

2) Mouse experiments:

Figure 4: a very small experimental cohort in figure 4A and 4B is noted (four mice in each group only). It is surprising that this produces a statistically significant result. It would be useful if the authors would be able to add additional experimental animals, which may have been generated in the meantime. A single round of injections may just result in chance outcome, even though the data "fit" the hypothesis and correlate with the human situation.

Figure 4B: please show an additional image of the interface between tumour and host brain to illustrate the invasiveness of the tumour.

Please show a staining of Sox9 of the cerebellar tumours displayed in figure 4B and D.

3) Clarification:

Page 8: the authors state The regulation of Sox 9 levels occurs on post transcriptional level, i.e. by phosphorylation and subsequent degradation. This is regulated by FB W7. On page 8, it is reported that group 3 and group 4 tumours showed lower Sox9 expression whilst SHH cases show a higher expression of Sox9 mRNA. How is this explained? How is the transcription of Sox9 regulated?

Page 9: Please provide reference for MB002 cells in the main manuscript. A brief (and adequate) explanation, and adequate reference is given in the supplementary material but the readership would benefit from this information being integrated into the main text.

Page 10 (top paragraph): "The RNA-Seq data revealed that all 3 analysed MB002 samples could be classified as group 3 tumours", this appears a circular argument to me when MB002 cells are known to be derived from group 3 tumours. Please clarify.

Editor's comments:

As our editorial policies allow for only a single round of major revisions, I should point out that it will be essential to carefully respond to all raised points during this round. Particular attention should be given to the concerns of referees 1 and 3 related to the in vivo data, their statistical significance and possible bias inherent in them. Another important point relates to referee 1's issues with the mechanistic experiments in Figures 1 and 2, which would need to be strengthened through additional work. Moreover, the overall impact of the manuscript for the field would clearly be increased by extending current in vitro experiments on Fbw7/Sox9 roles in resistance to therapy to in vivo settings, and providing some more insight into the underlying mechanisms (see referees 1 and 2). I will not repeat the remaining points in detail here, since they are well-explained in the referee reports, but would like to encourage you to get back to me with any specific questions/comments arising from the reports. Finally, please take note of the EMBO Journal article format guidelines, in particular please make sure to include a sufficiently informative Material and Methods section in the main manuscript.

Thanks for forwarding the reviews and offering us the opportunity to modify our manuscript for resubmission to EMBO journal. We appreciate that the Reviewer's find our study interesting and the data presented to be of high quality and suitable for publication in EMBO J. We agree with the Reviewer's that additional experiments addressing the FBW7/SOX9 pathway in medulloblastoma pathogenesis would strengthen our study. We have tried our best to accomplish this within the relatively short time frame considering work involved.

We have now performed a more thorough analysis of medulloblastoma cells/tumors in terms of proliferation, differentiation, apoptosis and metastasis in culture and in vivo, in response to cisplatin treatment as well as following induction of SOX9 or FBW7. In addition, we have expanded our analysis of the RNAseq data and performed comprehensive gene ontology and GSEA and now demonstrate that stabilization of SOX9 effectively promote EMT and pro-metastatic processes in medulloblastoma cells, supporting the in vivo and clinical findings.

With respect to the mechanistic experiments as well as the underlying mechanism(s) of how SOX9 stabilization promotes resistance to therapy, we feel that a complete investigation of the molecular mechanism is beyond the scope of the present study. However, we agree with the Reviewer's request that these are important issues to address in more detail and have therefore chosen to focus our resources on experiments that would cover new ground and increase the impact of this work. Specifically, we present new data related to how FBW7 mediate SOX9 degradation in response to cisplatin treatment and importantly, how SOX9 stability influence expression of genes attributed cisplatin resistance in primary medulloblastoma cells. This include, but is not limited to upregulation of the copper transporter ATP7A and downregulation of dual specific phosphatase DUSP2.

In summary, as you will see, we have addressed all the different points raised by the Reviewer's either by additional experiments and/or clarification/discussion and made the alterations in the manuscript accordingly. We thank the reviewers for their suggestions that we feel have led to a much-improved manuscript and hope that the paper will now be considered acceptable both in terms of being convincing and having sufficient impact.

REFEREE #1:

This paper from the Sangfelt lab shows that the Sox9 transcription factor is degraded by the SCF-Fbxw7 ubiquitin ligase, and describes a role for these interactions in the pathogenesis, and possibly the treatment of, medulloblastomas associated with SHH pathway activation. The identification of Sox9 as an Fbxw7 substrate initially fell out of a proteomic screen. While I have some specific comments below, in general the data support the idea that Sox9 can behave as a canonical Fbxw7

substrate in which Sox9 degradation is dependent upon its consensus degron and phosphorylation by GSK-3.

The paper next moves on to the physiologic role of Fbxw7-mediated degradation in medulloblastoma pathogenesis, migration and invasion. The choice of medulloblastoma is quite interesting, in light of the common Fbxw7 mutations found in this disease, and the role of Sox9 in this disease. While the data support the conclusions in this specific system, because these models rely on Sox9 and/or Fbxw7 overexpression, I feel they fall short of demonstrating a true physiologic role for Sox 9 stabilization by Fbxw7 mutations in naturally occurring medulloblastomas. Finally, because AKT and GSK3 are antagonistic, the paper examines the role of AKT inhibitors in therapy. This has always seemed like logical approach for Fbxw7-associated cancers, given the near-universal role of GSK3 in instigating substrate degradation, but has typically not been successful. These data look more promising, at least with respect to Sox9 abundance. However, the mechanisms underlying the synthetic lethality between reduced Sox 9 abundance and cisplatin is totally mysterious, and the specific role of Sox9 degradation in mediating the affect of the AKT inhibitor is shown in only a single overexpression experiment in supplemental Fig. 5. Overall I suspect that the identification of Sox9 as a bona fide Fbxw7 substrate is correct, but the physiologic significance of this pathway in medulloblastoma pathogenesis is less convincing.

General response.

We are glad that the reviewer finds this an interesting study with data supporting the conclusion that SOX9 is a canonical FBW7 target substrate. Unfortunately, exploring endogenous SOX9 levels upon FBW7 mutational inactivation in naturally occurring medulloblastomas is difficult to accommodate without available genetic models. Regardless, considering the various aspects of the cellular models used in this study and how they reinforce the conclusions made, our results argues for a true physiological role of SOX9 in medulloblastoma pathogenesis. We fully agree with the reviewer that investigating the mechanism underlying the synthetic lethality between SOX9 abundance and cisplatin is an important issue that we have now addressed in detail as specified point by point below.

Specific Points.

Fig. 1. The data are clear that Sox9 and Fbxw7 interact, and that GSK3 phosphorylates Sox9 on T236. Panel F is a little confusing, because efficient Fbxw7 binding usually requires both CPD phosphorylation, and GSK3 does not appear to phosphorylate T240. What is the affinity of this interaction? Is T240 phosphorylated? If not, is the binding independent of T240? Finally, is the GSK3 T236 phosphorylation primed by T240 phosphorylation? This appears not to be the case, and has important implications for understanding the impact of the AKT inhibitors, which has differential affects on primed versus non-primed GSK3 substrates.

We apologize for the confusion in this case. In fact, Fig 1F only assay the interaction between SCF-FBW7 and SOX9 with or without prior phosphorylation by GSK3 *in vitro*. In general, the FBW7 binding pocket make contacts with both degron phosphorylations and phosphorylation at position +4 may increase its binding affinity to some substrates (Welcker 2003 Mol Cell, Hau et al 2007, Mol Cell). Although the Figure EV1C demonstrates that binding of SOX9 by FBW7 requires an intact degron sequence (as shown for many other substrates), including the T240 residue, it is difficult to assess the affinity of FBW7 and SOX9 phosphorylated specifically on T240 compared to T236 without T240 specific antibodies. However, since our phospho-SOX9 antibody detects T240A but not T236A (EV1D), these data do suggest that T236 phosphorylation is not primed by T240 phosphorylation (EV1D), supporting the conclusion that GSK3-mediated phosphorylation of T236 can occur in the absence of T240 phosphorylation. However, this does not exclude the possibility that there may be other sites and kinases involved in priming GSK3 phosphorylation on T236 and stimulating FBW7-mediated SOX9 degradation.

Fig. 2. These data nicely show that endogenous Fbxw7 regulates endogenous SOX9 stability in 293 cells, which I presume is the cell type in which the original proteomics were performed?

HEK293 cells were used as another model to validate our proteomic data which was generated from HCT116 cells (FBW7 KO vs WT cells) and validated in Figures EV1A and EV2A.

2D. Why is the abundance of Fbxw7 so different in the various con-transfections? It is hard to determine a specific lack of degradation of the phosphorylation site mutants when Fbxw7 abundance seems much lower in these lanes.

We acknowledge the thorough review of this result and the remark regarding the different levels of expression in Figure 2D. We have repeated this experiment several times to ensure equal levels of FBW7 expression and now include new representative cycloheximide chase data. As shown in the new Fig 2D, FBW7 almost completely eradicates WT-SOX9 compared to the SOX9 mutants which are much more resistant to FBW7 overexpression.

2G. This experiment is flawed because the IP-western is done in the direction that would simply detect any ubiquitylate protein bound to Sox (including Fbxw7 itself), as opposed to showing Sox9-ubiquitin conjugates. The IP should be done in the other direction to show specific Sox9 immunoreactive conjugates, and also with mutant Fbxw7, as another control.

We apologize for not making this clearer in the manuscript. The cell lysates were prepared under denaturing conditions (1% SDS) to disrupt noncovalent interactions for in vivo ubiquitylation assays. Extracts were subsequently diluted in RIPA buffer prior to SOX9 immunopurification using SOX9-specific antibody and SOX9 poly-ubiquitin conjugates were detected by immunoblotting with anti-HA (ubiquitin) and anti-SOX9 antibodies. Thus, the high-molecular-weight smear in the previous Figure 2G represents polyubiquitylated SOX9. Nevertheless, to accommodate the reviewer's concern regarding additional controls, we have now repeated this assay with arginine mutant (R465A) and F-box deleted (dF) FBW7. The new data are shown in new Figure 2G and included in the manuscript (page 7). We have also carried out ubiquitin-immunoprecipitations followed by SOX9 immunoblotting, however, this approach gives a much lower quality of the data most likely due to high non-specific background (data not shown).

2H. The background in this in vitro experiment seems quite high (in the lane without Sox9). An important control, the use of mutant Fbxw7, is also missing.

In our experience, in vitro ubiquitylation assays using various FBW7 substrates often yield some background signal most likely representing auto-ubiquitylation of FBW7 and/or ubiquitylation of residual proteins bound to the beads. Thus, we would like to point out that this assay clearly demonstrates that there is significant ubiquitylation only when SOX9 is present in the reaction (lane 3, Figure EV 2H), suggesting that the ubiquitin smear primarily represent poly-ubiquitylated SOX9. We agree with the reviewer that the use of mutant FBW7 as another control could be useful, unfortunately we were unable to include this particular control due to the lack of commercially available mutant recombinant FBW7 (producing and purifying mutant FBW7 together with the other core ligase components in insect cells would be a very major undertaking). Instead, we have repeated the in vitro reactions using a different protocol (now described in materials and methods) with additional controls (e.g. dF-FBW7). The result from this experiment verifies the previous in vitro data and is now presented as new Figure 2H. Additionally, the new in vivo ubiquitylation data (new Figure 2G) includes mutant FBW7 as requested by the reviewer.

Fig 3. This figure, which shows that Sox9 is an Fbxw7 substrate in medulloblastoma cells, is much less convincing than the 293 cell experiments.

We agree with the reviewer's point that FBW7 overexpression is less effective in reducing endogenous SOX9 abundance in DAOY cells compared to HEK293 cells. However, we do show that knockdown of FBW7 stabilize endogenous SOX9 in several different cell types including MB DAOY cells (Figure EV 2C-D), and consistent with our hypothesis, that PI3K/AKT inhibitors further reduce SOX9 protein upon FBW7 induction (Figure 7A). Thus, one reason for the modest effects of FBW7 overexpression on endogenous SOX9 abundance in medulloblastoma cells might be due to increased PIK3/AKT pathway activity in these cells (Guerreiro *et al.*, 2008). Importantly, the data in Figure 7A & B and Figure EV 5B support this model and show that targeting the PIK3 pathway further destabilize SOX9 protein whereas FBW7 knockdown rescue SOX9 degradation.

Fig 2B. The difference in these survival curves may be significant, but is very modest and these data could reflect any Fbxw7 substrate(s) other than Sox9.

We agree with the reviewer that the survival data may also reflect dysregulation of other FBW7 substrates. This is now more carefully discussed in the manuscript (page 19).

3D. These overexpression assays are not sufficient to show that Fbxw7 degrades Sox9 in medulloblastoma cells. The authors should consider using the same knockdown approach to study endogenous proteins they used in 293 cells

Related to the point above (Figure 3), we in fact did the correct experiment suggested by the reviewer to show that depletion of FBW7 by siRNA increases SOX9 abundance and stability (Figure EV 2C and Figure 7B). In addition, the differences between HEK293 and medulloblastoma cells for SOX9 turnover might be due to various reasons as these cells are derived from different tissues (kidney vs brain) and different cell types (immortalized vs. tumor cells) which inevitably affects the cellular composition and interaction between proteins, for instance through increased RTK/PIK3/AKT pathway activity as suggested.

Fig. 4. Although the survival curve advantage in 4A is modest, I do think this figure overall supports the idea that Fbxw7 modulates pathogenesis and survival in these two Sox9-overexpression driven systems. However, if one accepts the idea that overexpression Fbxw7 degrades overexpressed Sox9 in medulloblastoma cells, I find these approaches somewhat contrived, since it seems almost certain that these results would be obtained. The data do not show a role for Sox9 stabilization in a medulloblastoma with a natural Fbxw7 mutation.

We acknowledge the reviewer's comment that our experimental set-up do not directly demonstrate that mutational inactivation of FBW7 stabilize SOX9 and promote medulloblastoma pathogenesis. An alternative model to assess the role of FBW7 mutation on SOX9 stability would be to generate mutant FBW7 knock-in medulloblastoma cells using the CRISPR-CAS9 system, however, this was not possible considering the time constraints for re-submission. Furthermore, a mutant FBW7 model would still be limited due to the implicit difficulties of separating SOX9 effects from other FBW7 substrates. Therefore, although the criticism that we use overexpression systems is valid, we nonetheless make a very strong case using these readouts. Thus, we believe we have taken this as far as we can using the established models and assays including analysis of FBW7-insensitive SOX9 mutant cells.

Fig. 6. These data do show a modest sensitization platinum sensitivity cause by Sox9 overexpression, and an even more reversal of this toxicity by Fbxw7 overexpression. Again the system seems set up just show just this affect.

There are two issues here. First, it was not certain if SOX9 expression causes treatment resistance in medulloblastoma cells and this is an important experiment to see if this is actually the case with or without FBW7. Second, it is clinically relevant as cisplatin is used in standard MB treatment and we feel that these assays are important to address cisplatin treatment failure in medulloblastoma patients with functional inactivation of FBW7 (or PIK3/AKT pathway mutations).

What are the supposed mechanisms through which Sox9 abundance mediates the synthetic lethality between platinum and AKT inhibition?

This is a very relevant point and we thank the reviewer for the opportunity to bring in new important data to improve the manuscript. During the re-submission process, we have performed additional experiments to investigate this further, and now provide data demonstrating that the synthetic lethality between cisplatin and PI3K/AKT inhibition is directly linked to SOX9 degradation (new Figure 7). To explore in more detail how SOX9 stabilization contribute to cisplatin resistance in medulloblastoma cells we first re-examined our RNAseq profiling data in MB002 cells (Figure 5) and cross-checked for differentially expressed genes previously reported to confer resistance against cisplatin (Galluzzi *et al.*, 2012, Lin *et al.*, 2011). As shown in new Figure 6D, expression of WT-SOX9 and stable SOX9 mutant differentially upregulated and repressed several cisplatin-resistance related genes. These results were validated by quantitative PCR and immunoblotting of a few select genes, including SOX9 mediated induction of ATP7A and repression of DUSP2 protein (new Figure 6D-E). In an attempt to further explore how SOX9 abundance affect the synthetic lethality between cisplatin and PIK3/AKT inhibition, we reasoned that targeting this pathway using inhibitors would counteract the SOX9-induced changes in ATP7A and DUSP2, rendering the medulloblastoma cells more sensitive to the treatment. Indeed, as shown in new Figure 7E, the combination treatment significantly attenuated SOX9-induced ATP7A expression and SOX9-repressed DUSP2 expression. In agreement with these findings, the increased sensitivity to cisplatin upon induction of FBW7 α in Daoy-expressing SOX9 (Figure 6B) was also associated with reduced expression of ATP7A and increased DUSP2 protein levels (new Figure 7G). Notably, in accordance with its function as a negative regulator of ERK1/2 activity, we found that SOX9 induction significantly increased ERK phosphorylation, and importantly, that the combination treatment reduced ERK phosphorylation in WT-SOX9 cells but to a much lesser extent in the SOX9 degron mutant cells (Figure 7E). While additional experiments including forced expression of DUSP2 and/or depletion of ATP7A in SOX9 overexpressing cells would further strengthen the mechanistic data, those experiments are not trivial given the challenge to transduce primary neuronal MB002 cells.

6D. Nice! This pretty clearly shows AKT-modulation of Fbxw7-driven Sox9 turnover.

Thank You.

6F. These data do not show that Sox9 stability is involved with the synthetic lethality. The only data that speak to this are in EV5D, but the amount of Sox9 overexpression isn't shown. Do normal amounts of Sox 9 expression (that is resistant to Fbxw7) also rescue the synthetic lethality?

We now provide additional drug response data and molecular profiling of MB002 cells expressing either the wild-type or stable mutant SOX9 (T236/240A), treated with cisplatin alone or with combination of cisplatin + AZD2014 (Figure 7E & F). Importantly, expression of mutant SOX9 (following doxycycline treatment) significantly attenuates the synthetic lethality for the drug combination and reduces cell death (as indicated by cleaved PARP) in comparison to expression of WT-SOX9 (Fig 7E & F).

REFeree #2:

SOX9 is a transcription factor expressed in the majority of solid tumors where it plays various roles depending on tumor type such as regulating cell invasion, differentiation and survival. However, little is known about how post-translational modifications regulate SOX9 function.

In this manuscript, Suryo Rahmanto et al. demonstrate that SOX9 interacts with FBW7, an ubiquitin ligase. Using biochemical experiments in vitro, the authors showed that FBW7 recognizes phosphorylated SOX9 by GSK3b at a conserved residue. This interaction leads to ubiquitination and degradation of SOX9. Using mutagenesis, they show that mutations of this phosphorylation site leads to Sox9 stabilization.

Later, Suryo Rahmanto and colleagues uses gene expression profile from primary medulloblastoma and show that FBW7 levels inversely correlate with patient survival of medulloblastoma patients, particularly in the SHH subgroup. Interestingly, whole exome sequencing analysis show that FBW7 is mutated in 11% of SHH medulloblastoma. Using tissue microarray, the authors show that while SOX9 and FBW7 mRNA levels do not correlate, there's an inverse correlation between SOX9 and FBW7 proteins levels within medulloblastoma.

Using inducible FBW7 construct together with SOX9 gain of function in orthotopic transplant of medulloblastoma cell line, the authors show that FBW7 overexpression decreases tumor spreading and migration, resulting in enhanced mice survival. Using Sox9 gain of function in medulloblastoma initiation cells (MIC), they show that SOX9 dramatically increases metastasis and migration of these cells. This increase is reduced by FBW7 overexpression. They also observe a correlation between SOX9 levels and metastasis in medulloblastoma patients and show that SOX9 overexpression promotes the expression of genes involved in EMT.

In addition, they show that overexpression of SOX9 in medulloblastoma cell lines reduces efficiency of cisplatin treatment. Interestingly, this can be counteracted by inhibition of the PI3K/AKT axis which promotes GSK3 activity and therefore SOX9 degradation.

Overall the experiment described in this manuscript are well conducted and controlled. The findings reported brings important insights about the role of Sox9 posttranslational modifications in cancer progression and response to therapy. While we believe that this manuscript will appeal the readers of EMBO J, we think the manuscript can be further improved by addressing the following points prior to publication:

General response.

We are encouraged by the reviewer's view that our manuscript "brings important insights about the role of Sox9 posttranslational modifications in cancer progression and response to therapy that will appeal the readers of EMBO J". The reviewer points out two important issues. One relates to the role of SOX9 degradation and its effect on processes linked to medulloblastoma pathogenesis. The other relates to the mechanistic aspects of how SOX9 stabilization promote cisplatin resistance (as also pointed out by reviewer #1).

1. While the observations regarding tumor growth and mice survival are well characterized (Fig 4) it is not clear what's happening at the cellular level. For instance, how are cell death, differentiation, proliferation and invasion affected by perturbation of SOX9, FBW7 or inhibitors

treatment? (both in orthotopic transplant and in vitro. The authors should study in more detail these parameters by immunofluorescence.

We thank the reviewer for the suggestion to assess various cellular parameters upon perturbation of SOX9 by FBW7 and/or inhibitors treatment. We have now characterized in more detail the cellular response of Daoy–SOX9 cells upon dox-induction of FBW7 by metabolic assay alamar blue (Figure EV 4B), as well as cell death indicators (Figure EV 4C) by immunoblotting. Dox-induction of FBW7 (and thus degradation of SOX9) restored Daoy-SOX9 doubling time from 35 hours to 30 hours (EV4B). Immunoblotting of cell death markers including cleaved PARP and cleaved caspase 3 showed essentially no change upon FBW7-mediated suppression of SOX9 in these cells (Figure EV 4C). Consistently, IHC staining of fixed tumor tissues using Ki67 and cleaved caspase 3 antibodies revealed a moderate increase in cell proliferation (Ki67 staining) without obvious induction of apoptosis (Cl. Casp3 staining, not shown) in the dox-treated Daoy-SOX9 / T-FBW7 α tumors (as shown in Figure EV 4 D-E). Note, all of the tumors were examined at the end point of tumor progression, when mice had to be sacrificed. At this point, our data showed that the increased amount of metastases/dissemination in the spinal cord and forebrain is causing a significantly shorter tumor latency and death in these animals (4A-B and Figure EV 4E-H). Collectively, our data suggest that SOX9 promotes increased malignancy and significantly shorter survival in vivo due to increased cell motility and tumor spread of DAOY cells as compared to FBW7 expressing tumors where SOX9 is degraded.

Related to the effects of the inhibitors on SOX9 protein turnover and cell viability we first analyzed changes in SOX9 protein and mRNA upon treatment with cisplatin, AZD2014, and the combination of both inhibitors in MB002 cells. Our data from this set of experiments demonstrated that both AZD2014 and a Cisplatin / AZD2014 combination treatment promoted SOX9 degradation. This is indicated by the enhanced depletion of total SOX9 protein with the combination treatment in MB002 cells (Figure 7C). Importantly, depletion of SOX9 protein occurs while both *SOX9* and *FBW7* mRNAs were significantly elevated (Figure EV 5C), whereas the SOX9 stable mutant remained elevated in MB002 cells treated with this combination (Figure 7E). Combined Cisplatin and AZD2014 treatment clearly increased PARP and caspase 3 cleavage in MB002 cells whereas expression of SOX9-WT or the SOX9 stable mutant markedly reduced cytotoxicity (as assessed by both cleaved PARP and resazurin-based cell viability assay (Fig 7E & F). (Fig 7C). Thus, together with our viability data included in the manuscript (Fig 6F), we conclude that the cisplatin / AZD2014 combination treatment is cytotoxic and not cytostatic toward MB002 cells. Unfortunately, as the AZD2014 drug is unable to pass over the blood brain barrier (according to communication with the drug company) we could not perform in vivo experiments using this drug combination. Also, considering the relatively short time frame of the animal work involved, the next step in this project will be to perform detailed in vivo analysis using different combinations of drugs able to pass the blood-brain barrier but this will of course be a very major undertaking which is beyond the scope of the present manuscript.

2. The microarrays analysis are superficially presented. The analysis seems to focus only on few selected genes. The authors should perform Gene ontology analysis and GSEA with previously published data sets analyzing EMT, Sox9 target genes, and any other relevant data sets.

We agree with the reviewer and have now performed more comprehensive analysis of the transcriptional profiling data in MB002 cells, including gene set overlap analysis with GSEA signatures as presented in Table EV 4 as well as a targeted GSEA against signatures related to EMT, metastasis and migration as presented in (Table EV 5 & Figure EV 5B). The results from this analysis is now included in the manuscript (result, discussion and materials and method sections). Importantly, in agreement with the findings in Figure 5D-G, the GSO analysis reveals a significant overlap with Hallmark EMT genes and the GSEA analysis further demonstrate significant enrichment of cancer related EMT factors and pro-metastatic genes. Notably, enrichment of these

signatures with expression of SOX9-CPD stable mutant as compared to SOX9-WT strongly imply that stabilized SOX9 more effectively promote these cellular processes (Figure 5D,G, Figure EV 5B, Table EV 4, Table EV 5). Strikingly, cross-referencing differentially expressed genes with SOX9 ChIP studies identified a significant number of putative SOX9 target genes significantly differentially expressed between MB002 cells expressing SOX9-WT and the FBW7-resistant SOX9 mutant, which further overlapped with pro-metastatic genes (Figure 5G). As transcriptional profiling was performed after 8 hours of dox-treatment it is likely that we would observe even stronger effects on these processes at later time points. This is also confirmed by the more pronounced effects of VIM and SLUG after 24 hours (Figure 5E-F).

3. The role of FBW7 in regulating cisplatin sensitivity has already been described (Song et al. 2015; Yu et al, 2013). This work must be cited.

This work is now cited.

4. Regarding the role of Sox9/Fbw7 in the resistance to therapy, all the experiments presented in the manuscript are performed in vitro. It would be nice if the authors can perform these experiments in vivo.

We have now extended these treatment studies with *in vivo* data. We have performed an experiment related to Figure 6 in order to further evaluate the role of SOX9 on cisplatin resistance. We orthotopically transplanted normal DAOY control cells or DAOY cells with overexpressed SOX9-WT and SOX9-T236/240A into nude mice's and treated them with vehicle or with cisplatin for 14 days. The results from this *in vivo* experiment are summarized in the results (see Figure 6C and Figure EV 6A) and show significant cisplatin treatment efficacy only in DAOY parental cells (see Figure 6C, black vs red lines) where the treatment increases the overall survival as compared to SOX9-WT or SOX9-T236/240A DAOY cell xenografts where cisplatin treatment did not show any significantly increased survival. SOX9-WT cells partially responded to cisplatin treatment but in the T236/240A mutant there was not even a trend of increasing survival from cisplatin treatment, supporting the results demonstrating enrichment of genes linked to cisplatin resistance upon SOX9 expression/stabilization (Figure 6D).

5. While it seems clear that FBW7 low levels/SOX9 high levels are involved in resistance to cisplatin, the mechanism still remains unclear. Could the author provide some experiments/discussion about how SOX9 would promote resistance to therapy in this case?

We have now performed additional experiments to address the mechanism in more detail to determine how FBW7/SOX9 influence cisplatin sensitivity. These data are now included as part of Figure 6 and 7 in the revised manuscript. In brief, we showed that SOX9 expression modulated expression of a panel of genes linked to cisplatin resistance including the induction of the copper efflux transporter ATP7A and repression of the negative regulator of ERK1/2 pathway DUSP2 following SOX9 expression in MB002 medulloblastoma cells. Please also see a more detailed response referring to this point above, rev#1, point 6.

6. The model presented on Figure 7 seems largely speculative. While the upper part as well as the targeted degradation is well supported by the data, the experimental support for the lower part is much less clear. For instance, the EMT-like reprogramming and stem cell phenotype are poorly supported by the data. This should be removed.

We thank the reviewer for this suggestion and we have now modified the model (new Figure 8 in the revised manuscript) and removed the "stemness phenotype". Given our new data for

SOX9 stabilization on EMT, migration and cisplatin resistance, we maintained these phenotypes in the model.

REFEREE #3:

The authors present data suggesting a role of FBW7 and Sox9 in growth and metastatic spread of medulloblastoma. Whilst FBW7 is downregulated in all subgroups of medulloblastoma, there is a high incidence of FBW7 mutations in the SHH subgroup of medulloblastoma. A number of experiments demonstrate the role of FBW7 in degrading Sox9, and conversely, a stabilisation of Sox9 by down-regulation or inactivation of FBW7. The work is logically structured and presents data in a concise and intuitive fashion. First, the authors demonstrate Sox9 interaction with FBW7 through a conserved motif, phosphorylation by GSK3 and experiments demonstrating a Sox9 ubiquitination and degradation independent of GSK3. Comparison of FBW7 expression profiles across databases of primary medulloblastomas shows an inverse correlation of expression with clinical outcome. These data are further corroborated by testing expression levels of FBW7 and Sox9 mRNA and Sox9 protein on a set of 142 medulloblastomas. In parallel, xenografting of cells with modulated FBW7 expression shows that down regulation of FBW7 promotes metastatic spread of tumour cells. A borderline significance is seen in the survival. Generally, the quality of the data is excellent or very high, and the conclusions are supported by the data. There are a number of minor issues that should be addressed by the authors:

Main issues:

1) TMA analysis: clarify how the mRNA levels were quantified? From the materials section, it appears that arbitrary scoring was performed, which is not adequate, in particular if no scoring criteria (number of stained particles, number of nuclei containing particles etc) were defined. An adequate methodology using digital image quantification on TMA sections should be used. For example, The RNAscope technology enables a fully quantifiable readout using professional image analysis methods, including those offered by the company itself.

General response: We are glad that the reviewer finds our study to be well-executed with high quality data supporting the conclusions made.

Specific response: We apologize for the limited information regarding the scoring criteria. TMAs were scored manually due to unavailability of appropriate software to analyze multiplex RNA signals at the time of the study. It was only from our recent communication with the Advanced Cell Diagnostic, the supplier of the RNA scope, that we were made aware of Halo Software (developed by Indica Lab), which may be used to perform RNA multiplex analysis. In our view, a digital quantification using a software can work well in many cases but it can also generate potential biases. We think it is important to look at all TMA stainings manually (in a blinded way) in order to justify patterns, stainings and potential artefacts / indirect signals created. Accordingly, we have now included detailed information about scoring criteria in the Materials & Methods section. As an example of the stainings and scoring, we have attached representative pictures to this document (see below) and in Figure EV 3D. Briefly, scoring of TMAs was performed blindly based on fluorescent signal intensity by 2 individuals without previous knowledge about subgroup and by evaluating one fluorescent signal at a time in order to not create any biases.

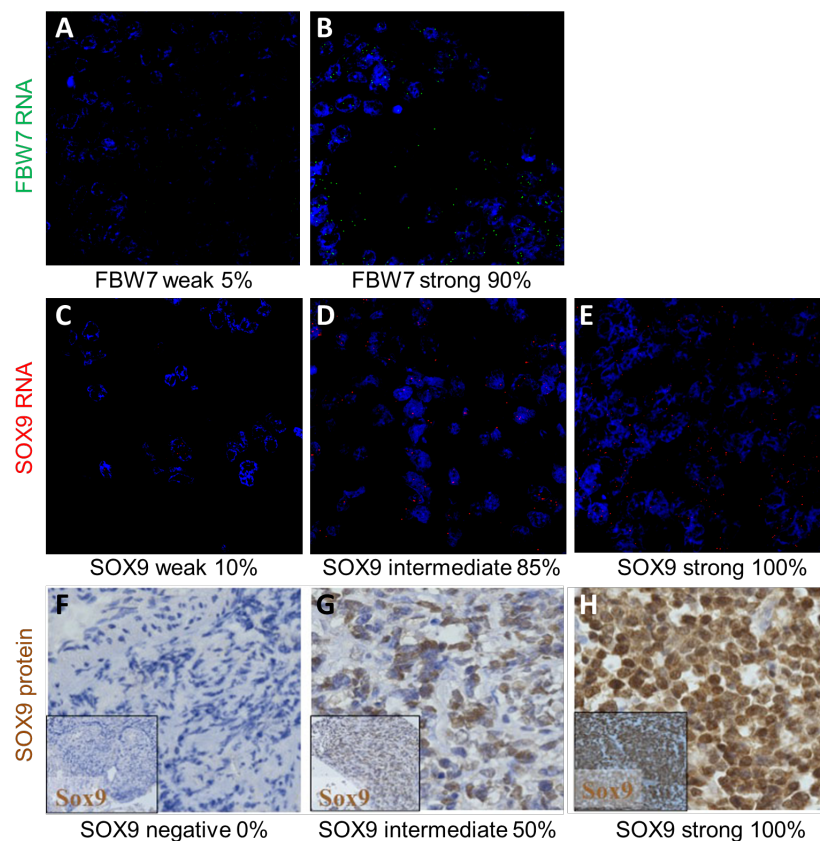


Figure 1. Representative examples of various TMA sections stained for FBW7 RNA (A-B), SOX9 RNA (C-E) as well as SOX9 protein (F-H).

Furthermore, the quantification of the Sox9 immunostaining on TMA sections is not specified. Again, it is essential that this is done using a method to generate quantitative data, which have the benefit of generating continuous and unbiased data. It is further recommended that the authors use an additional Sox9 antibody to corroborate and validate their IHC data. The values/readout of the 2 antibodies should be compared/combined.

We understand the reviewer's concern and have now specified how the quantification of SOX9 stainings was performed in greater detail in the Materials & Methods – “Immunohistochemistry and RNA scope assay on tissue sections and TMAs”. We are well aware of the potential biases of using a single antibody in IHC to study protein expression on TMA slides. As the TMAs used for these experiments are very limited and the FFPE blocks have been exhausted

during DNA extractions we were unable to obtain additional TMA sections from our co-authors (Drs. Andrey Korshunov and Stefan Pfister) in Heidelberg.

To minimize the potential pitfall of using a single antibody, we initially performed preliminary IHC staining using 3 different SOX9 antibodies (AB3075 from R&D, AB5535 from Millipore, and AB3697 from Abcam) on representative TMA sections obtained from human brain tumor sections that we previously characterized to be enriched with SOX9-positive cancer cells (see Figure 2 below (Swartling *et al.*, 2012)). From these preliminary experiments, we observed very similar SOX9 staining patterns with the AB3075 and AB5535 antibodies. The AB3697 antibody showed a slightly more diffuse staining with less variance between different cells in the tumor as compared to the other antibodies. We therefore selected the SOX9 antibody from Millipore (AB5535) that we also previously used for IHC on human sections (see Figure 5G in Swartling *et al.* Cancer Cell 2012). Again, we apologize for not being able to perform additional analysis using different SOX9 antibodies on all the TMAs.

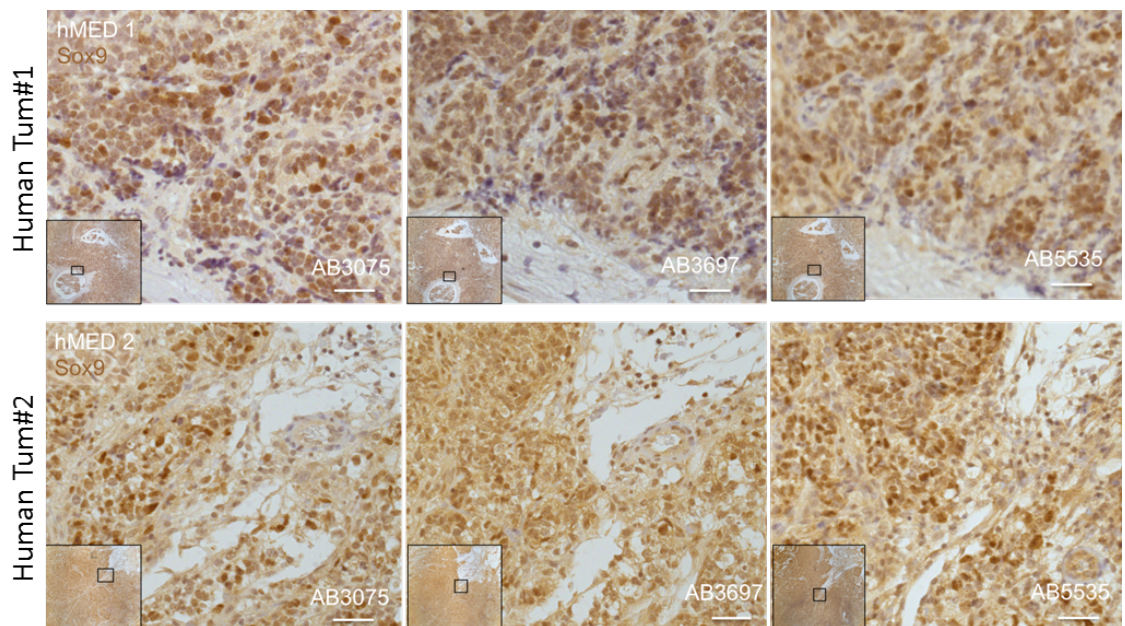


Figure 2. IHC staining of SOX9 protein by AB3075 (R&D), AB3697 (Abcam), and AB5535 (Millipore) in 2 representative human brain tumors, which known to be enriched with SOX9-positive cancer cells

Another important rationale for a more advanced image analysis method for the 142 samples is the potential value in correlating the Sox9 levels with the clinical outcome (metastasis and spread).

In this cohort of 142 specimens we find that SOX9 protein levels do correlate with clinical outcome in regards to metastasis (M3 stage) at diagnosis (see Figure 4H). In order to visualize all patient data and the scoring of our samples we have now included Appendix “Table RNA Scope and clinical data” that includes more detailed patient information and SOX9 scoring correlated to clinical outcome.

*TMA*s are generated from various primary sources which may vary in fixation time, generating a potentially heterogeneous set of *TMA* cores. Using an antibody derived from a different clone may mitigate some of the problems of *TMA*. How was the variability of IHC staining accounted for? Does it explain some of the discrepancies to clinical outcomes as shown in the figure?

We fully agree with the reviewer that various primary tumor sources may vary and generate a potentially heterogeneous set of *TMA* scores. The RNA probes for FBW7 and SOX9 are also used in an in situ fashion that would create a similar intratumoral bias with variation in fixation time as an antibody would create. However, as mentioned above we did perform initial IHC experiments using different antibodies on brain tumor samples with consistent SOX9 staining patterns (see Figure with SOX9 staining above). Although SOX9 positive staining could give a false positive result in some instances, we have previous experience of analysing SOX9 staining and knowledge of how an intense SOX9 staining with a significant proportion of nuclear staining appears on *TMA*s (Swartling et al. Oncogene 2009). In our set of *TMA*s we did not observe any significant proportions of strongly expressed cytoplasmic SOX9 staining. We experienced rather low SOX9 staining in many *TMA* samples but also found clear examples of elevated SOX9 protein in the nucleus without changes in SOX9 RNA levels. Thus, if we had many false positives we would expect to find high scores with samples showing elevated SOX9 levels both at RNA and protein level. We indeed identified a few SOX9 negative *TMA* samples. This would give a 1:1 score and thus not further influence the correlation analysis. All in all, it is possible that our findings rather underestimate the extent of SOX9 positives.

Figure 3F: what are the different colours in figure 3F indicate?

The four colours indicate a specific molecular subgroup for each sample (Blue, red, yellow, and green represent the WNT, SHH, the Group 3, and 4). Samples with non-identifiable molecular signature are shown in gray circles. We have now indicated this information in the Figure Legend 3F.

2) Mouse experiments:

Figure 4: a very small experimental cohort in figure 4A and 4B is noted (four mice in each group only). It is surprising that this produces a statistically significant result. It would be useful if the authors would be able to add additional experimental animals, which may have been generated in the meantime. A single round of injections may just result in chance outcome, even though the data "fit" the hypothesis and correlate with the human situation.

We agree with the reviewer that it would be useful to increase the number of animals and we actually performed another round of injections of 12 additional mice. However, during the course of this experiment we discovered that the doxycycline was not effective due to a mix-up between a fresh and an expired batch of doxycycline food. This was evident because in a parallel experiment using our doxycycline-regulated GTML mice in where we can follow brain tumor growth with bioluminescence (Swartling et al. Genes&Dev, 2010), the bioluminescent levels were significantly higher (10^2 times) and not completely suppressed (10^4 - 10^5 times) as we experience when these mice are treated with doxycycline (Swartling et al., 2010). Unfortunately, as the old dox-food could have severely compromised the dox-induced expression of FBW7 in Daoy cells, and consequently the survival data, we were forced to surrender this new cohort of animals. Although the criticism that we use a small cohort in Figure 4A is valid, we nonetheless make a strong case considering the other data presented in the manuscript.

Figure 4B: please show an additional image of the interface between tumour and host brain to illustrate the invasiveness of the tumour. Please show a staining of Sox9 of the cerebellar tumours displayed in figure 4B and D.

We thank the reviewer for the suggestion and have now included representative stainings for the Daoy-SOX9/T-FBW7 α tumors as supplementary data for the manuscript (Figure EV 4E-H). The SOX9 staining near the central regions of the Daoy tumors were generally lower in the dox-receiving group in comparison to the untreated (Figure EV 4F & H, indicated by black arrows). Interestingly, we found no differences in the SOX9 staining intensity at the tumor interface between the 2 groups of Daoy-SOX9/T-FBW7 α tumors (Figure EV 4G & H). To this end, we speculate that the high SOX9 levels at the tumor interface in the dox-treated group may be due to reduced dox exposure in such region (i.e. tumour grow surrounding blood vessel and expanding outward).

With regards to the tumors presented in Figure 4D, we had reported the SOX9 staining (and other relevant differentiation markers) in our previous study in Cancer Cell (Table S1, (Swartling *et al.*, 2012); selected parts of the table shown below). The MIC and MIC-SOX9 brain tumors were previously referred as P0C Tumors (T1-4) and SOX9 Tumors (T1-5), respectively. We hope this information provides sufficient background for the experiments.

Table S1. Immunostaining patterns in N-MYC-induced brain tumors

Tumor (no.)	Injected in	Mis-placed	GFAP	Nestin	SOX9	OLIG2	SYP	OLIG3	KCNA1	β -catenin (nuclear)	0	No expression
P0C (T4)	Cerebellum	No	1	1	0	0	2	0	1	0	1	0-25% of cells
P0C (T1)	Cerebellum	No	0	1	1	2	1	1	2	0	2	25-50% of cells
P0C (T3)	Cerebellum	No	1	1	1	1	2	0	1	0	3	50-75% of cells
SOX9 (T1)	Cerebellum	No	2	2	4	4	1	1	0	0	4	75-100% of cells
SOX9 (T3)	Cerebellum	No	2	2	3	3	1	0	0	0	4	75-100% of cells
SOX9 (T5)	Cerebellum	No	2	2	4	3	1	1	0	0	4	75-100% of cells

3) Clarification:

Page 8: the authors state The regulation of Sox 9 levels occurs on post transcriptional level, i.e. by phosphorylation and subsequent degradation. This is regulated by FBW7. On page 8, it is reported that group 3 and group 4 tumours showed lower Sox9 expression whilst SHH cases show a higher expression of Sox9 mRNA. How is this explained? How is the transcription of Sox9 regulated?

Several studies have demonstrated transcriptional upregulation of SOX9 by a number of factors or pathways in various malignancies. The WNT- β catenin (Larsimont *et al.*, 2015), the SHH-BMP4 (Wang *et al.*, 2010), the EGFR-NFATc1 (Chen *et al.*, 2015), the NOTCH-1 (Capaccione *et al.*, 2014), the YAP1 (Song *et al.*, 2014), and the NF-kB (Sun *et al.*, 2013) are among a few examples. In medulloblastoma, the higher expression of SOX9 in the WNT and SHH in comparison to the group 3 and 4 are possibly due to of aberrant activation of the WNT and the SHH pathway (as we previously described in Swartling *et al.* Cancer Cell, 2012).

Page 9: Please provide reference for MB002 cells in the main manuscript. A brief (and adequate) explanation, and adequate reference is given in the supplementary material but the readership would benefit from this information being integrated into the main text.

We thank the reviewer for the suggestion. We have now included a brief description regarding MB002 cells (Bandopadhyay *et al.*, 2014) in the main manuscript.

Page 10 (top paragraph): "The RNA-Seq data revealed that all 3 analysed MB002 samples could be classified as group 3 tumours", this appears a circular argument to me when MB002 cells are known to be derived from group 3 tumours. Please clarify.

We apologize for the confusion and we wish to clarify that our intention is to highlight that forced expression of SOX9-WT or its stable CPD mutant did not alter the molecular subgrouping of

MB002 from Group 3 to another (presumably WNT or SHH subgroup in where SOX9 levels are indeed higher (as just discussed in the previous point 3 above)). We felt that it is important parameter to assess since we had previously shown that activation of SOX9 not only marks but might also promote a SHH-dependency in medulloblastoma cells originating from MYCN–transduced mouse neural stem cells (Swartling *et al.*, 2012). Obviously, SOX9 overexpression cannot force switching one malignant subgroup (Group 3) into another (e.g. SHH). Hopefully, the updated text referring to Figure 5A makes more sense now.

References:

Bandopadhyay P, Bergthold G, Nguyen B, Schubert S, Gholamin S, Tang Y, Bolin S, Schumacher SE, Zeid R, Masoud S, Yu F, Vue N, Gibson WJ, Paoletta BR, Mitra SS, Cheshier SH, Qi J, Liu KW, Wechsler-Reya R, Weiss WA *et al.* (2014) BET bromodomain inhibition of MYC-amplified medulloblastoma. *Clin Cancer Res* 20: 912-25

Capaccione KM, Hong X, Morgan KM, Liu W, Bishop JM, Liu L, Markert E, Deen M, Minerowicz C, Bertino JR, Allen T, Pine SR (2014) Sox9 mediates Notch1-induced mesenchymal features in lung adenocarcinoma. *Oncotarget* 5: 3636-50

Chen NM, Singh G, Koenig A, Liou GY, Storz P, Zhang JS, Regul L, Nagarajan S, Kuhnemuth B, Johnsen SA, Hebrok M, Siveke J, Billadeau DD, Ellenrieder V, Hessmann E (2015) NFATc1 Links EGFR Signaling to Induction of Sox9 Transcription and Acinar-Ductal Transdifferentiation in the Pancreas. *Gastroenterology* 148: 1024-1034 e9

Galluzzi L, Senovilla L, Vitale I, Michels J, Martins I, Kepp O, Castedo M, Kroemer G (2012) Molecular mechanisms of cisplatin resistance. *Oncogene* 31: 1869-83

Guerreiro AS, Fattet S, Fischer B, Shalaby T, Jackson SP, Schoenwaelder SM, Grotzer MA, Delattre O, Arcaro A (2008) Targeting the PI3K p110alpha isoform inhibits medulloblastoma proliferation, chemoresistance, and migration. *Clin Cancer Res* 14: 6761-9

Larsimont JC, Youssef KK, Sanchez-Danes A, Sukumaran V, Defrance M, Delatte B, Liagre M, Baatsen P, Marine JC, Lippens S, Guerin C, Del Marmol V, Vanderwinden JM, Fuks F, Blanpain C (2015) Sox9 Controls Self-Renewal of Oncogene Targeted Cells and Links Tumor Initiation and Invasion. *Cell Stem Cell* 17: 60-73

Lin SC, Chien CW, Lee JC, Yeh YC, Hsu KF, Lai YY, Lin SC, Tsai SJ (2011) Suppression of dual-specificity phosphatase-2 by hypoxia increases chemoresistance and malignancy in human cancer cells. *J Clin Invest* 121: 1905-1916

Song S, Ajani JA, Honjo S, Maru DM, Chen Q, Scott AW, Heallen TR, Xiao L, Hofstetter WL, Weston B, Lee JH, Wadhwa R, Sudo K, Stroehlein JR, Martin JF, Hung MC, Johnson RL (2014) Hippo coactivator YAP1 upregulates SOX9 and endows esophageal cancer cells with stem-like properties. *Cancer Res* 74: 4170-82

Sun L, Mathews LA, Cabarcas SM, Zhang XH, Yang AC, Zhang Y, Young MR, Klarmann KD, Keller JR, Farrar WL (2013) Epigenetic Regulation of SOX9 by the NF-kappa B Signaling Pathway in Pancreatic Cancer Stem Cells. *Stem Cells* 31: 1454-1466

Swartling FJ, Grimmer MR, Hackett CS, Northcott PA, Fan QW, Goldenberg DD, Lau J, Masic S, Nguyen K, Yakovenko S, Zhe XN, Gilmer HC, Collins R, Nagaoka M, Phillips JJ, Jenkins RB,

- Tihan T, Vandenberg SR, James CD, Tanaka K *et al.* (2010) Pleiotropic role for MYCN in medulloblastoma. *Genes Dev* 24: 1059-72
- Swartling FJ, Savov V, Persson AI, Chen J, Hackett CS, Northcott PA, Grimmer MR, Lau J, Chesler L, Perry A, Phillips JJ, Taylor MD, Weiss WA (2012) Distinct neural stem cell populations give rise to disparate brain tumors in response to N-MYC. *Cancer Cell* 21: 601-13
- Wang DH, Clemons NJ, Miyashita T, Dupuy AJ, Zhang W, Szczepny A, Corcoran-Schwartz IM, Wilburn DL, Montgomery EA, Wang JS, Jenkins NA, Copeland NA, Harmon JW, Phillips WA, Watkins DN (2010) Aberrant epithelial-mesenchymal Hedgehog signaling characterizes Barrett's metaplasia. *Gastroenterology* 138: 1810-22
- Bandopadhyay P, Bergthold G, Nguyen B, Schubert S, Gholamin S, Tang Y, Bolin S, Schumacher SE, Zeid R, Masoud S, Yu F, Vue N, Gibson WJ, Paoella BR, Mitra SS, Cheshier SH, Qi J, Liu KW, Wechsler-Reya R, Weiss WA *et al.* (2014) BET bromodomain inhibition of MYC-amplified medulloblastoma. *Clin Cancer Res* 20: 912-25
- Capaccione KM, Hong X, Morgan KM, Liu W, Bishop JM, Liu L, Markert E, Deen M, Minerowicz C, Bertino JR, Allen T, Pine SR (2014) Sox9 mediates Notch1-induced mesenchymal features in lung adenocarcinoma. *Oncotarget* 5: 3636-50
- Chen NM, Singh G, Koenig A, Liou GY, Storz P, Zhang JS, Regul L, Nagarajan S, Kuhnemuth B, Johnsen SA, Hebrok M, Siveke J, Billadeau DD, Ellenrieder V, Hessmann E (2015) NFATc1 Links EGFR Signaling to Induction of Sox9 Transcription and Acinar-Ductal Transdifferentiation in the Pancreas. *Gastroenterology* 148: 1024-1034 e9
- Galluzzi L, Senovilla L, Vitale I, Michels J, Martins I, Kepp O, Castedo M, Kroemer G (2012) Molecular mechanisms of cisplatin resistance. *Oncogene* 31: 1869-83
- Guerreiro AS, Fattet S, Fischer B, Shalaby T, Jackson SP, Schoenwaelder SM, Grotzer MA, Delattre O, Arcaro A (2008) Targeting the PI3K p110alpha isoform inhibits medulloblastoma proliferation, chemoresistance, and migration. *Clin Cancer Res* 14: 6761-9
- Larsimont JC, Youssef KK, Sanchez-Danes A, Sukumaran V, Defrance M, Delatte B, Liagre M, Baatsen P, Marine JC, Lippens S, Guerin C, Del Marmol V, Vanderwinden JM, Fuks F, Blanpain C (2015) Sox9 Controls Self-Renewal of Oncogene Targeted Cells and Links Tumor Initiation and Invasion. *Cell Stem Cell* 17: 60-73
- Lin SC, Chien CW, Lee JC, Yeh YC, Hsu KF, Lai YY, Lin SC, Tsai SJ (2011) Suppression of dual-specificity phosphatase-2 by hypoxia increases chemoresistance and malignancy in human cancer cells. *J Clin Invest* 121: 1905-1916
- Song S, Ajani JA, Honjo S, Maru DM, Chen Q, Scott AW, Heallen TR, Xiao L, Hofstetter WL, Weston B, Lee JH, Wadhwa R, Sudo K, Stroehlein JR, Martin JF, Hung MC, Johnson RL (2014) Hippo coactivator YAP1 upregulates SOX9 and endows esophageal cancer cells with stem-like properties. *Cancer Res* 74: 4170-82
- Sun L, Mathews LA, Cabarcas SM, Zhang XH, Yang AC, Zhang Y, Young MR, Klarmann KD, Keller JR, Farrar WL (2013) Epigenetic Regulation of SOX9 by the NF-kappa B Signaling Pathway in Pancreatic Cancer Stem Cells. *Stem Cells* 31: 1454-1466

Swartling FJ, Grimmer MR, Hackett CS, Northcott PA, Fan QW, Goldenberg DD, Lau J, Masic S, Nguyen K, Yakovenko S, Zhe XN, Gilmer HC, Collins R, Nagaoka M, Phillips JJ, Jenkins RB, Tihan T, Vandenberg SR, James CD, Tanaka K *et al.* (2010) Pleiotropic role for MYCN in medulloblastoma. *Genes Dev* 24: 1059-72

Swartling FJ, Savov V, Persson AI, Chen J, Hackett CS, Northcott PA, Grimmer MR, Lau J, Chesler L, Perry A, Phillips JJ, Taylor MD, Weiss WA (2012) Distinct neural stem cell populations give rise to disparate brain tumors in response to N-MYC. *Cancer Cell* 21: 601-13

Wang DH, Clemons NJ, Miyashita T, Dupuy AJ, Zhang W, Szczepny A, Corcoran-Schwartz IM, Wilburn DL, Montgomery EA, Wang JS, Jenkins NA, Copeland NA, Harmon JW, Phillips WA, Watkins DN (2010) Aberrant epithelial-mesenchymal Hedgehog signaling characterizes Barrett's metaplasia. *Gastroenterology* 138: 1810-22

2nd Editorial Decision

02 August 2016

Thank you again for submitting your revised manuscript for our consideration. It has now been assessed once more by two of the original referees, whose comments are copied below. As you will see, referee 2 considers their original concerns adequately addressed, and referee 3 is satisfied with the response to their original concerns about small mouse cohorts. However, the latter reviewer still retains serious concerns regarding quantification of RNA and protein levels and their correlation with patient survival, and questions the decisiveness of these data for supporting strong conclusions. Given that I had already stressed this point in my original decision letter, I feel it would still be important to address these points by attempting to strengthen the image analysis and presenting better expression-patient outcome-correlations, following referee 3's comments and suggestions. I am therefore returning the manuscript to you once more for an additional round of minor revision, to allow you to deal with this remaining concern.

I hope you will be able to make the required additional revisions and submit a re-revised version as soon as possible, and that we should then be in the position to swiftly proceed with acceptance and publication of this study in The EMBO Journal.

REFeree COMMENTS

Referee #2:

The authors adequately addressed the comments raised during the initial review. The paper is now suitable for publication.

Referee #3:

In my critique to the authors work I have asked to address 2 major issues. One of the issues was related to the detection of RNA and protein by RNA scope and immunohistochemical staining, respectively. These tests were done on a TMA. My concerns were mainly the rather arbitrary determination of signal in the 142 samples, and I queried a correlation to the patient survival.

Another point of criticism was the very small number of experimental animals that were used to make a claim of a role of FBW 7 in survival.

The authors have partially addressed my concern regarding the quantification and were unable to address the issue with the small experimental animal numbers.

Although it remains unsatisfactory to make a relatively strong claim based on a very small number of experimental animals, I can see the technical and organisational difficulties they experienced in repeating the experiments. I therefore can accept this response.

The authors have further responded to the point of criticism regarding the quantification of the RNA and protein levels and distribution in the TMA. I am not convinced about the assessment that a purely semi-quantitative, rather arbitrary evaluation yields the same accuracy and objectivity as an image analysis system. My concerns are further confirmed when I look at the images the authors give to illustrate the assessments. Arguably, any of these "numbers" associated with the figures would find very little agreement across a random selection of observers, yielding a very significant interobserver variability (in particular the figures for the FBW7 and Sox 9 RNA ISH. For example, how do the authors arrive at the numbers stated here for C, D, E?

The logic of the scoring table (avoiding multiplication with 0 and therefore generating an RANK table) is not entirely clear to me. Perhaps I am missing the point, but I cannot identify a problem by multiplying one of the 2 scores by 0. If one of the 2 parameters is 0, then the overall score will automatically be 0.

The figures contain a number of arrows, but does not clear what the arrows are pointing at.

"In this cohort of 142 specimens we find that SOX9 protein levels do correlate with clinical outcome in regards to metastasis (M3 stage) at diagnosis (see Figure 4H). In order to visualize all patient data and the scoring of our samples we have now included Appendix "Table RNA Scope and clinical data" that includes more detailed patient information and SOX9 scoring correlated to clinical outcome".

Whilst this explanation is more satisfactory than previously, I find the integration of figure 4H into multiple other figures related to mouse experiments puzzling. Also, the authors have not indicated what the cut off level for Sox 9 "high" and "low" would be. Overall, I feel this has been addressed to some extent, but relatively superficially. The graph is not particularly convincing.

2nd Revision - authors' response

12 August 2016

Referee #3:

In my critique to the authors work I have asked to address 2 major issues. One of the issues was related to the detection of RNA and protein by RNA scope and immunohistochemical staining, respectively. These tests were done on a TMA. My concerns were mainly the rather arbitrary determination of signal in the 142 samples, and I queried a correlation to the patient survival. Another point of criticism was the very small number of experimental animals that were used to make a claim of a role of FBW 7 in survival.

The authors have partially addressed my concern regarding the quantification and were unable to address the issue with the small experimental animal numbers. Although it remains unsatisfactory to make a relatively strong claim based on a very small number of experimental animals, I can see the technical and organisational difficulties they experienced in repeating the experiments. I therefore can accept this response.

Response:

We thank the reviewer for his / her understanding regarding our technical difficulties in repeating the animal xenograft study in a limited time.

The authors have further responded to the point of criticism regarding the quantification of the RNA and protein levels and distribution in the TMA. I am not convinced about the assessment that a purely semi-quantitative, rather arbitrary evaluation yields the same accuracy and objectivity as an image analysis system. My concerns are further confirmed when I look at the images the authors

give to illustrate the assessments. Arguably, any of these "numbers" associated with the figures would find very little agreement across a random selection of observers, yielding a very significant interobserver variability (in particular the figures for the FBW7 and Sox 9 RNA ISH. For example, how do the authors arrive at the numbers stated here for C, D, E?

Response:

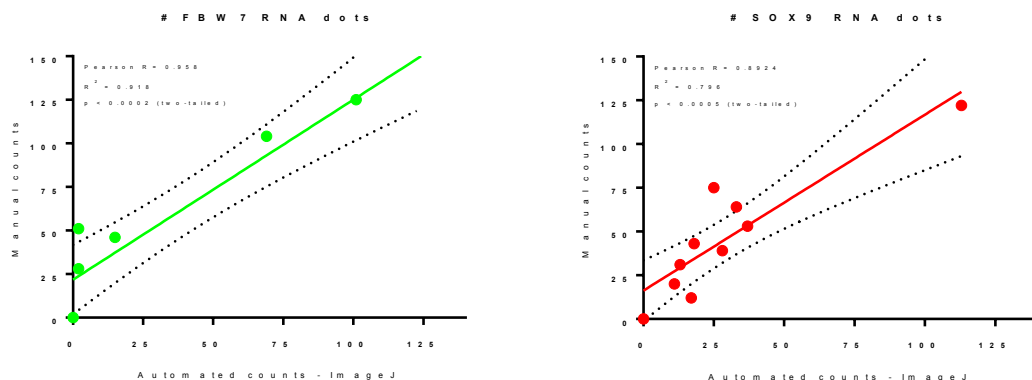
We believe that the reviewer's concern is partly based on a miscommunication and we apologize that we did not provide enough details how the manual quantification was performed in the previous response letter. To clarify this issue further we now provide a more precise scoring protocol (as outlined below) and hope this clarifies our methods of assessment

- The TMA array was stained with the specified RNA fluorescent probes and DAPI and examined using ZEISS confocal microscopy for fluorescent signals. In the microscope, specific RNA signals appear as discrete, bright fluorescent spots in or around the DAPI-stained nuclei (Figure EV 3D). To score "RNA intensity" in each tumor specimen we quantified the number of RNA spots in each positive cell (indicating the relative numbers of RNA molecules per cell). The total number of RNA fluorescent spots was manually counted from at least 3 different image fields by 2 independent, blinded individuals, followed by adjustment to the number of DAPI-stained and RNA fluorescent signal-positive nuclei. RNA intensity was scored 0 for negative, 1 for low (less than 3 spots/cell) to 3 (more than 10 spots/cell) accordingly. Similarly, the "percentage of tumor cells expressing the RNA" was analysed by estimating the proportion DAPI-stained nuclei with a positive RNA fluorescent signal in a given field (i.e. 0 – 100%). The average percentage (or RNA positivity) was calculated and likewise converted to scores; 0 for negative, 1 for low (less than 20%), 2 for intermediate (20-80%) and 3 for high (more than 80%) To grade the overall RNA expression in a given tumor specimen, the "RNA intensity" score was multiplied with the score of the proportion of RNA positive cells, generating the possible expression scores of 0, 1, 2, 3, 4, 6 or 9. To this end, and to ensure linear distribution, the resulting multiplication scores were converted to "ranks" from 1 to 7, respectively.

This assessment is now illustrated in greater detail in the new sample images in Fig EV 3D.

- The SOX9 protein expression was analysed by immunohistochemistry as previously described (Swartling *et al.*, 2012). Similar to RNA, an overall SOX9 protein score was calculated and converted to rank. Finally, the level of SOX9 expression was calculated by dividing SOX9 protein rank with the SOX9 RNA rank (Figure 3F).

Further, to assess the objectivity of our manual assessment, we have now decoded several images and performed automatic digital quantification using ImageJ. For automated analysis, a macros script was written for ImageJ (v 1.51e, NIH) to count fluorescent RNA spots with a size of 2 micron or greater. In so doing, we observed significant and robust correlations between our manual and automated analysis (Pearson R-correlation value of 0.89 – 0.96), arguing for non-biased assessment and scoring (Response Figure 1).



Response Figure 1. Pearson correlation analysis of FBW7 (Left) and SOX9 RNA (Right) counts from automated (x-axis) and manual (y-axis) image analysis.

The reason why we did not attempt digital image quantification on TMA sections in the first place is because blinded manual assessment (although semi-quantitative) is generally sufficient to grade expression on TMAs. In our opinion, automated evaluation needs careful standardization and is also prone to errors due to the inability to handle non-ideal situations, such as inadequate sample preparation, heterogeneous tissues, cells that appear merged, nonspecific signal or damaged tissue morphology, etc. Further, because medulloblastoma tumors have intra-tumoral heterogeneity, evaluation of SOX9 expression on TMAs is particularly challenging.

The logic of the scoring table (avoiding multiplication with 0 and therefore generating an RANK table) is not entirely clear to me. Perhaps I am missing the point, but I cannot identify a problem by multiplying one of the 2 scores by 0. If one of the 2 parameters is 0, then the overall score will automatically be 0.

The figures contain a number of arrows, but does not clear what the arrows are pointing at.

Response:

Regarding the logic of the scoring table, the reviewer is correct that multiplying one of the 2 scores by 0 is not a problem.

However, to account for the total SOX9 protein / RNA ratio, it is not possible to calculate the ratio in the case of RNA score being 0 (negative). At this point, we believe that the “SOX9 protein / RNA” ratio needs to be taken into account given the variability in the levels of SOX9 transcript across different medulloblastoma molecular subgroups. Specifically, it is known that the *WNT* and the *SHH*- subgroup is known to have an elevated level of SOX9 mRNA transcript (Swartling *et al.*, 2012), with this probably as a result of activation of the *WNT* or *SHH* pathway.

Thus, by translating score (0-9) to rank (1-7), a total SOX9 protein/RNA ratio can be measured. In order to better exemplify our TMA analysis we now provide a new figure detailing scoring and ranking for SOX9 and FBW7, with circles showing some RNA spots and arrows pointing to SOX9 (protein) positive cells in the new Figure EV3D.

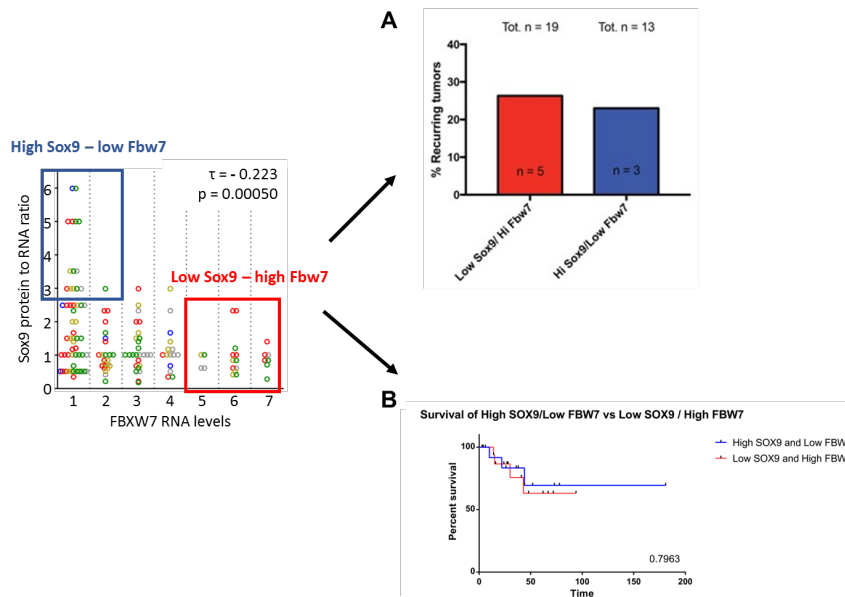
"In this cohort of 142 specimens we find that SOX9 protein levels do correlate with clinical outcome in regards to metastasis (M3 stage) at diagnosis (see Figure 4H). In order to visualize all patient data and the scoring of our samples we have now included Appendix "Table RNA Scope and clinical data" that includes more detailed patient information and SOX9 scoring correlated to clinical outcome".

Whilst this explanation is more satisfactory than previously, I find the integration of figure 4H into multiple other figures related to mouse experiments puzzling. Also, the authors have not indicated what the cut off level for Sox 9 "high" and "low" would be. Overall, I feel this has been addressed to some extent, but relatively superficially. The graph is not particularly convincing.

Response:

We agree with Reviewer 3 that still more clinical (survival) correlations would constitute an important advance. However, it should be emphasized that the cohort in question is small (142 patients) and comprised of all four different medulloblastoma subtypes that are rather diverse with regard to overall patient survival. Furthermore, since there are multiple confounding factors (*e.g.* age, MYC/MYC*N* amplification, chromosome 17q amplification/gain, p53 mutation/chromothripsis (in some of the subgroups), beta-catenin mutation, metastasis at diagnosis and radio-/chemotherapy-induced secondary malignancies etc) known to influence the survival rate of medulloblastoma patients, strong correlations are not easily observable with such a limited material.

Nevertheless, we have tried our best to fulfill this request by performing additional expression-outcome analysis using the available data set. Tumor specimens were classified as “high SOX9 / low FBW7” or “low SOX9 / high FBW7” using the median as a cut-off (Response Figure 2). No statistically significant difference was found between these groups with respect to patient overall survival or tumor recurrence (Response Figure 2). Thus, no clear conclusions could be drawn from this cohort in terms of overall survival. This has been described in the result section on page 9 and if requested these data can also be added as supplementary results.



Response Figure 2. Tumor recurrence (A) and Overall Survival (B) analysis between 19 medulloblastoma patients expressing Low SOX9/ High FBW7 versus 13 patients expressing High SOX9 / Low FBW7.

Furthermore, the reason why SOX9-metastasis data (Figure 4H) was incorporated in Figure 4 was mainly because it directly relates to the other findings demonstrating increased metastasis/migration upon SOX9 overexpression. The correlation of SOX9 protein expression with metastasis (M stage) was obtained following comparison of patients with high SOX9 protein RANK to patients with low SOX9 protein RANK (rank 1-3 against 5-7; $p=0.038$). Statistical significance was evident also with more stringent cut-offs (e.g. rank 1-2 vs 6-7; $p=0.031$).

Finally, we wish to emphasize that our intention with this work was not to provide a complete investigation of the clinical significance of FBW7/SOX9 expression in medulloblastoma patients as such analysis is beyond the scope of the present study.

Nonetheless, we exemplify that it is important to study SOX9 and FBW7 expression in clinical material and that SOX9 is indeed correlating with low FBW7 levels in a diverse and rather small set of childhood brain tumors.

Reference:

Swartling FJ, Savov V, Persson AI, Chen J, Hackett CS, Northcott PA, Grimmer MR, Lau J, Chesler L, Perry A, Phillips JJ, Taylor MD, Weiss WA (2012) Distinct neural stem cell populations give rise to disparate brain tumors in response to N-MYC. *Cancer Cell* 21: 601-13

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Dr. Fredrik J. Swartling & Dr. Olle Sangfelt

Journal Submitted to: EMBO J

Manuscript Number: EMBOJ-2016-93889

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if $n < 5$, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
 - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
 - are tests one-sided or two-sided?
 - are there adjustments for multiple comparisons?
 - exact statistical test results, e.g., P values = x but not P values $< x$;
 - definition of "center values" as median or average;
 - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

USEFUL LINKS FOR COMPLETING THIS FORM

<http://www.antibodypedia.com>
<http://1degreebio.org>
<http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo>

<http://grants.nih.gov/grants/olaw/olaw.htm>
<http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm>
<http://ClinicalTrials.gov>
<http://www.consort-statement.org>
<http://www.consort-statement.org/checklists/view/32-consort/66-title>

<http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tur>

<http://datadryad.org>

<http://flicshare.com>

<http://www.ncbi.nlm.nih.gov/gap>

<http://www.ebi.ac.uk/ega>

<http://biomodels.net/>

<http://biomodels.net/miriam/>
<http://ij.biochem.sun.ac.za>
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
<http://www.selectagents.gov/>

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	In general, we balance the sample size to achieve power to detect statistically significant effects. For in vitro experiments, eg involving protein measurements, our long term experience on the number of biological repeats needed to ensure statistical power, additionally statistical guidelines for each individual test is followed.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample size was chosen based on the minimum number of animals needed to obtain statistical power, all in accordance with the ethical regulations in Uppsala, Sweden and in line with the Principles of the 3R's (to refine, reduce and replace number of experimental animals used).
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Prior to in vivo experiments, experimental end point was set to 80 days post transplantation. Thus, remaining individuals were sacrificed after 80 days. Individuals who reached experimental endpoints as outlined in ethical protocol (ie. weight loss from chemotherapy treatment) was also excluded from analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Yes, when randomizing the treatment groups two researchers were involved in dividing the individuals in to the experimental groups, to prevent any selection bias.
For animal studies, include a statement about randomization even if no randomization was used.	Where applicable, mice were randomized into different groups after stereotaxic injection. Experimental groups for the treatment studies were randomized a week post injection, when treatment was initiated. An exception being mice injected with tumor cells bearing Tet-responsive constructs, these cells (and mice) were preconditioned with or without doxycycline three days prior to transplantation, therefore cohorts could not be randomized in the same manner.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Mice were monitored for symptoms by two researchers as well as technical staff at the animal facility daily/weekly throughout the experiments. Mice were monitored by multiple people and subsequent analysis was in part done by additional investigator not involved in the actual in vivo work.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Majority of the experiments required doxycyclin food (that was colored (red/blue) to make sure it was not mixed up with normal chow) and/or drug/vehicle administration, this made it difficult to keep the group identity blinded to investigators. But as stated above, mice were monitored by multiple people and subsequent analysis was in part done by additional investigator not involved in the actual in vivo work.
5. For every figure, are statistical tests justified as appropriate?	For various in vitro experiments (qPCR, cell viability, dose response curve, and migration assay) we performed Multiple comparisons of 2-way ANOVA with Bonferroni post test. The data are parametric, which justifies this statistical test. For correlation analyses, either Spearman rank correlation was used when data was parametric without requiring a normal distribution or Kendall's tau when ranked-data with ties was used, which are appropriate justifications for these tests. Other statistical tests were performed in concordance with the respective analysis tools (GSEA, GSO, cummeRbund for RNAseq differential expression) and assumed to be justified.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The raw values of the biological samples follow a normal distribution when plotted
Is there an estimate of variation within each group of data?	For the RNAseq data, agreement between replicates was estimated using Lin's concordance correlation coefficient. Of the 9 comparisons, 7 had a concordant correlation coefficient >0.97 and the remaining two had a concordant correlation >0.95. For various in vitro experiments variation are expressed either as standard deviation or standard error of the mean (SEM). For animal experiments Kaplan-Meier survival analysis was performed and analyzed as described in materials and methods.

Is the variance similar between the groups that are being statistically compared?	For the RNAseq expression data, variance within each group was calculated and the differences in variance between groups estimated to be less than 10%. For the remaining data, given the experiments were done in a very controlled manner, comparing parameters within the same experimental testing objects, we expect similar data variance
---	---

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Antibodies used in this study were obtained from various suppliers and are referenced with catalogue number in the Appendix document. All antibodies have been validated previously apart from rabbit polyclonal antibody specific against SOX9 pT236&240 which was produced by Innovagen AB which was tested for specificity in this study.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Daoy, D324MED, HCC1143, HEK293 and U343MG cell lines were obtained from the American Type Culture Collection (ATCC). HCT116-FBW7-KO cells have been provided by B. Vogelstein. M8002 cell line was obtained from Dr. Yoon-Jae Cho, Stanford Univ, USA and has been characterized recently in Bandopadhyay et al., 2014. Medulloblastoma-initiating cells (MIC) derived from postnatal, day 0, mouse cerebellar neural stem cells – transduced with T58A-NMYC alone or those which were further transduced with SOX9 (POCT/SOX9) come from a previously published study (Swartling et al., 2012). All cell lines tested negatively for mycoplasma infections by a standard PCR-based detection method. Testing was done on a regular basis.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Mus musculus, 6-9 week old athymic Nude - Foxn1nu mice (obtained from Harlan Laboratories, Netherlands). Mice were maintained at approved facilities in Uppsala, Sweden, using green-line cage systems with paper enrichment and food and water ad libitum.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Ethical protocols C144/13 and C128/15 were approved by the Regional ethical vetting committee in Uppsala, Sweden.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Compliance confirmed.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	See Point 12 below
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Decoded paraffin-embedded samples/TMAs from medulloblastoma patients were collected between 1995 and 2007 with informed consent as previously described in Korshunov et al JCO, 2010 and used in this report in collaboration with Drs. Stefan Pfister and Andrey Korshunov, Heidelberg, Germany. Experiments were conformed to the principles set out in the WMA Declaration of Helsinki and the Dept. of Health and Human Services Belmont Report. Further details of the samples are provided in Korshunov et al JCO, 2010 as cited in materials and methods.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The RNA-seq data generated in the study has been assigned as "GSE84320" and available from NCBI. Data can be accessed for a private view at this time using the following link: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=ividlicspgpyl&acc=GSE84320
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right)).	See point 18 above.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: Primary Data Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in <i>Shewanella oneidensis</i> MR-1. Gene Expression Omnibus GSE39462 Referenced Data Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CRA/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	We have included a Data Availability section in M&M in where such data is formally cited.
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biocompare (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A
---	-----