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Wnt/β-catenin signaling induces MLL to create epigenetic changes in salivary gland tumors

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 27 November 2012

Thank you for submitting your research manuscript (EMBOJ-2012-83727) to our editorial office. It has now been seen by three referees and their comments are provided below.

In general, all reviewers appreciate your study and are principally supportive of publication in The EMBO Journal. Nevertheless, they do raise a number of important concerns, and emphasize that a significant revision of the manuscript will be required. Although this will entail additional experimentation, many issues can be addressed by textual changes and clarifications that improve the intelligibility of the manuscript for the non-specialist reader. This is less of a concern for reviewer #1, as s/he is an expert in the field. Furthermore, both reviewer #2 and #3 suggest that the conclusions regarding the "cancer stem cell" character of the tumorigenic cell population you identify are either further substantiated or toned down. In that regard, it might be useful to clarify the rationale behind the specific markers used. Finally, both reviewers question the motivation for the tissue regeneration experiments in the context of this manuscript, and it might be worth considering if you find the data necessary to support the key conclusions of your paper.

Overall, I would like to invite you to submit a suitably revised manuscript to The EMBO Journal that addresses the concerns raised by the reviewers. I should add that it is our policy to allow only a single major round of revision and that it is therefore important to address all criticism at this stage. Please do not hesitate to contact me to further discuss the required revisions.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

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

REFEREE COMMENTS

Referee #1

This is an interesting and well written paper reporting on the interconnection between β-catenin and Bmp signaling in control of salivary gland cancer development through cancer stem cell populations. The findings are novel and have clinical implications, and should be of considerable general interest. Several issues need to be addressed.

Major points:

1) There are several sub-types of salivary gland squamous cell carcinomas, such as the mucoepidermoid and adenoid cystic types, with different gene expression profiles (Gibbons et al., 2001, The Laryngoscope 111, 1373-1378). The authors should specify the sub-types of the human samples that were analyzed (showed in Suppl. Table 1), and the type of salivary gland SCCs that develop in the mouse.
2) An important question is whether the K14-Cre transgene used for these studies is expressed in all or only a subset of epithelial cells in the salivary gland (acinar, ductal and/or myoepithelial cells), as other K14 Cre transgenes have been previously reported to target only a portion of epithelial cells in this gland (Jonkers, Nature genetics 29, 418-425). When does K14-Cre start to be expressed in these various populations during development?
3) Along these lines, and in view of recent work, for instance by the Kopan's laboratory on skin SCCs, it is important to know whether all tumor cells are K14 Cre positive and have undergone the expected gene rearrangements.
4) In the human relevance section (page 10), the authors used several human head and neck cancer cell lines rather than existing human salivary gland SCC lines, such as H292 or A-253, that, for the sake of consistency, should be included in the analysis.
5) It seems that in the double mutant mice, SCCs develop only in the submandibular gland. Did the authors also check the parotid glands? This is an important point, given the fact that, in human, most tumors arise in the parotid and not submandibular gland.

Minor points:

1. Figure 1A : β-catenin staining seems to be mostly in spindle-like cells. Double staining with keratin antibodies is required to distinguish tumor from stromal cells.
2. In Figure 1B, the authors claim that up to 75% of aggressive SGSCC are nuclear β-catenin positive and pSmads negative. Representative images need to be shown. How many sections/fields have the authors examined? What was the positive control for pSmad staining?
3. In Figure 3G, the CD24 and CK6 pattern of staining is very different in the sections from β-CatGOF versus β-CatGOF x Bmpr1aLOF mice. Images for the double mutant mice have much higher background levels and less green signal (β-catenin). Is it possible that the samples were not equally processed?
4. Figure 6 F,G : statistical significance of differences between samples plus/minus ICG needs to be assessed.
5. Supplemental Figure 1C : it looks as if hair follicles in the double mutant mice degenerate into cysts. Hence, how did the authors quantify hair follicle density?
6. Supplemental Figure 1E : K10 may not be a good marker for salivary gland cell differentiation, since, at least in normal mouse salivary glands, K10 is not expressed in acinar cells. Also, are K10 positive cells also K14 Cre positive?

Referee #2

This manuscript investigates the role of the wnt pathway in driving cancer stem cell proliferation, using salivary gland tumors as a model.

The introduction is over concise. A couple of lines explaining where the story is going and linking sentences to connect the paragraphs would help readers follow the train of thought. A diagram explaining the anatomy of the salivary gland and the location of the different cell populations would greatly assist the general reader.

Results

The authors begin by examining expression of b catenin and nuclear smad1/5/8 in head and neck squamous cell carcinoma (SCC) arising from the salivary glands and other locations. Nuclear b catenin is present in undifferentiated areas of the tumor and nuclear smad1/5/8 in differentiated areas, suggesting these staining patterns correlate with differentiation (Fig 1A). Are the images in Fig 1A serial sections of the same tumor (it is difficult to correlate the H and E with the immunohistochemical panels)? Where do the insets come from in the main panel? Scale bars are missing throughout Figure 1.

The correlation with grade (the criteria used to score tumors as grade 2 and 3 need to be clearly explained for non specialist readers) is consistent with the link between the marker expression and differentiation (Fig 1B). Given the heterogeneity of SCCs, both within individual tumors and between lesions it is essential to specify exactly the scoring criteria used for the immunostaining.

Fig 1C shows nuclear b catenin expression in CD44 and CD24 positive cells. Given the weak evidence for CD24 and CD44 being stem cell markers in SCC it might be better to describe them as "candidate stem cell markers". The scoring rules used to count percentages of double positive cells need to be given: are these percentages of all tumor cells or cells at the tumor front? The statement that "many" cells with nuclear b catenin express CD24/CD44 is confusing, given the low proportion (3-6%) of double positive cells in figure 1B.

The evidence presented on human SCC does not support the claim that "High Wnt/b-catenin and attenuated Bmp signals lead to head and neck squamous cell carcinomas in humans".

A genetic experiment in mice to investigate the effect of coexpression of a bcatenin gain-of-function mutant and null BMPr1a mutant in keratin14 positive cells is then described. It would be helpful to include a diagram summarizing these complex mutant animals and explain the pattern of Krt14 expression in the salivary gland. The image in Fig 1D is confusing as the lesion appears to arise from the mediastinum rather than the oral cavity. Supplementary Figure 1 lacks scale bars and images of control mice are needed in 1C to interpret the changes in the H and E panels. The double mutant animals die from salivary gland lesions by 3 months of age, whereas the single mutants have no effect on survival over this interval. It would be helpful to confirm that the mouse tumors have similar changes in b catenin and smad staining to those shown for the human tumors. In the main text changes in apoptosis and gene expression shown in Fig S1B are said to be at p1, whereas the figure legend refers to p90.

The authors then turn to analysis of CD24+ CD29+ cells. In Fig 2A it is essential to show isotype controls for each channel as the level of background staining may change significantly between normal and tumor samples. In the text it is stated that "In the tumors, CD24+CD29+ cells strongly clustered (Fig. 2B)". This is misleading. Fig 2B only shows Cd24 staining and even in tumors the proportion of double positive cells by FACS is under 10%. No evidence is provided that the double positive cells are clustered.

Staining of cytospins argues that the double positive cells from tumors are proliferating and generate growing tumors. It would be helpful to know the proportion of cells which are not double positive that express proliferation markers, to exclude the possibility that the markers simply enrich for proliferating cells rather than "cancer stem cells".

The focus then shifts to the role of the double positive cells in regeneration. There is no mention of how this was done in the methods section. Axin2cre/reporter mice reveal an upregulation of wnt signalling in regenerating glands in the intercalating duct cells. The single mutant mice regenerate more rapidly than controls and have a higher proportion of double positive cells (Fig 3). Validation of CK6 as a proliferation marker in each tissue is required, eg by comparing the proportion of BrdU positive cells in the CK+ and - cell populations. It is not clear how this work fits with the cancer stem cell theme and the manuscript might be better without it.

The manuscript then returns to cancer stem cells. A third surface marker, SSEA1 is found to be co-expressed with CD24 and CD29. Array analysis and Wnt inhibition demonstrates the expression of a set of Wnt responsive developmentally significant genes in the double positive cell population (Fig 4). Global changes in histone marks are also evident comparing double positive cells from single and double mutant mice. Similar changes are seen in Human SCC cell lines in vitro. In sphere forming cultures from double mutant mice wnt inhibition retards sphere growth, an effect blocked by HDAC inhibition (Fig 5). In figure 6 a role for MLL1 is investigated. Figure S5C is unclear...high power insets showing the channels separately are required. Interestingly, Mll1 or b catenin knockdown promoted differentiation, but these experiments would be strengthen by rescue of the phenotype with the relevant siRNA resistant constructs.

Overall
There is a large quantity of data here, but the manuscript is poorly structured and hard for a non specialist to follow. There are also some significant problems in the interpretation of the results. This work is not therefore suitable for the EMBO Journal in its present form, but could be substantially improved by adding additional explanation for the majority of readers who are unfamiliar with the salivary gland, removing material not directly relevant to the cancer stem cell theme and addressing the specific points above.

Referee #3

Wend and colleagues generate a double transgenic mouse model carrying a gain of function in beta-catenin and loss of function in Bmpr1a. These mice develop salivary gland tumors. The authors then isolate CD24/CD29 positive cell populations and show evidence that this population carries the cell of origin of the salivary tumors. Using a Wnt/beta-catenin inhibitor ICG-100 the authors can force these cancer cells towards differentiation. As a mechanism the authors suggest that Wnt/beta-catenin cause transcriptional permissive chromatin via MLL1.

The manuscript is dense and contains a tremendous amount of data. However, the rationale for the experiments is not always clear and it is difficult to understand what the original hypothesis of this work was and what precise conclusions can be drawn from the experiments. My main concern is that the paper does not show what the title suggests. (1) There is no direct evidence provided that Wnt/beta-catenin drives MLL1 activity in salivary glands tumors. (2) The actual evidence that the isolated CD24+/CD29+ tumor population is indeed the cancer stem cell population of this tumor is not sufficiently substantiated. All that is shown is that the CD24+/CD29+ tumor population can produce tumors in a transplantation experiment. I believe these claims either need to be supported by more data or the authors must tone down their claims throughout the manuscript.

However, the authors convincingly show that activation of beta-catenin and inhibition of Bmp signaling can cause salivary tumors in mice. These tumors contain a highly tumorigenic cell population (CD24+/CD29+), which can be forced towards differentiation using a Wnt/beta-catenin inhibitor. This part is interesting and in my opinion gets lost in the current version of the manuscript.

Major comments:

1. What is the relevance of beta-catenin activation and deletion of BMP signaling within the CD24/CD29 positive tumor cell population in mouse salivary tumors? The authors do not provide evidence for high nuclear beta-catenin and absence of phospho Smads in this population. Is the increase in proliferation within this population directly due to beta-catenin activation and inhibition

BMP signaling or is it indirect?
2. Figure 1: The rationale for testing nuclear beta-catenin co-localization with CD44 and CD24 in the context of the manuscript is unclear. The authors name them as stem cell markers and it is unclear onto what observations or publications the authors base this assumption with respect to squamous cell carcinomas or salivary glands.
3. Along the same line; one would probably expect a co-localization of nuclear beta-catenin with CD44 in the invading front of the tumor but the IF shown in figure 1C does not seem to present that. Furthermore, the number CD44/beta catenin co-localizing cells are not quantified and the authors do not state if there are any triple positive (beta catenin/CD24/CD44).
4. Why do the authors then chose to flow sort CD24+/CD29+ cell populations from the mouse tumors? And the statement that CD24+/CD29+ strongly clustered (Fig. 2B) is not reflected in the figure. CD29 staining is not shown and one would expect a general basement membrane staining.
5. For both mouse and human tumors the authors should show where the Bmpr1a is actually expressed and whether it overlaps with CD44, 24 or 29.
6. Figures 2F and G are unusual presentations of transplantation assays and should rather include the information of how many times the experiments have been performed and how often those resulted in the formation of a tumor.
7. Serial transplantation assay of the isolated cancer cell population is currently one of the gold standards to define a cancer stem cells population. The authors claim that the CD24+/CD29+ population gave rise in serial transplantation assays but do not provide the data.
8. The rationale for the wounding experiments is unclear. Just because the CD24/CD29 population can contribute to tissue regeneration does not qualify the cells as stem cells. It is also unclear onto what experimental results the authors build their statement that 'single mutant tissues regenerated faster'. The authors refer to figure 3C, which only shows that the number of K14+ proliferating cells increased. Whether normal tissue architecture has been achieved is not shown.
9. The overall increase in H3K4 trimethylation can be due to increased proliferation of an undifferentiated cell population but is not solely or uniquely altered in cancer stem cells.
10. Figure 5A; The authors test the effect of the beta-catenin inhibitor on cell proliferation of double mutant cells. The effect of the drug might be simply explained by negatively regulating cell proliferation. It would be interesting to see how cell proliferation is affected in the single mutant cells.
11. What happens to cellular localization of Mll1 when the cells are treated with CHIR?
12. The strong conclusion in the last paragraph are not supported by experiments: "We suggest that a beta-catenin / CBP / MLL1 complex drives self-renewal...". The authors only show in figure 6F and G that the overall level of H3K4 trimethylation in the promoters is lower when cells are treated with ICG-001. However, the cells differentiate in response to the drug and the reduced trimethylation levels might be the result of the increased contribution of the differentiated population, which has silenced the promoters. In other words, from the provided experiments one cannot distinguish between cause and consequence of H3K4 methylation changes at these sites.

Minor comments:

1. The manuscript would benefit from a more precise description of the figures, i.e. 'many nuclear beta-catenin-positive cells....(Fig. 1A-C)' does not seem to be reflected in the quantification when only less than 7% are double positive.

1st Revision - authors' response 25 March 2013

General response to all reviewers:

We would like to thank the reviewers for their critical and insightful reviews of our manuscript. We have now made a major revision of the manuscript and addressed the concerns raised. Whole sections were removed from our previously submitted manuscript, e.g. the regeneration experiment. Additionally, we have worked rigorously to streamline our story and focused on the editing of the MS for easier reading. Please find the point-by-point responses to the critiques of the reviewers.

Referee #1:

This is an interesting and well written paper reporting on the interconnection between β-catenin and Bmp signaling in control of salivary gland cancer development through cancer stem cell populations. The findings are novel and have clinical implications, and should be of considerable general interest. Several issues need to be addressed.

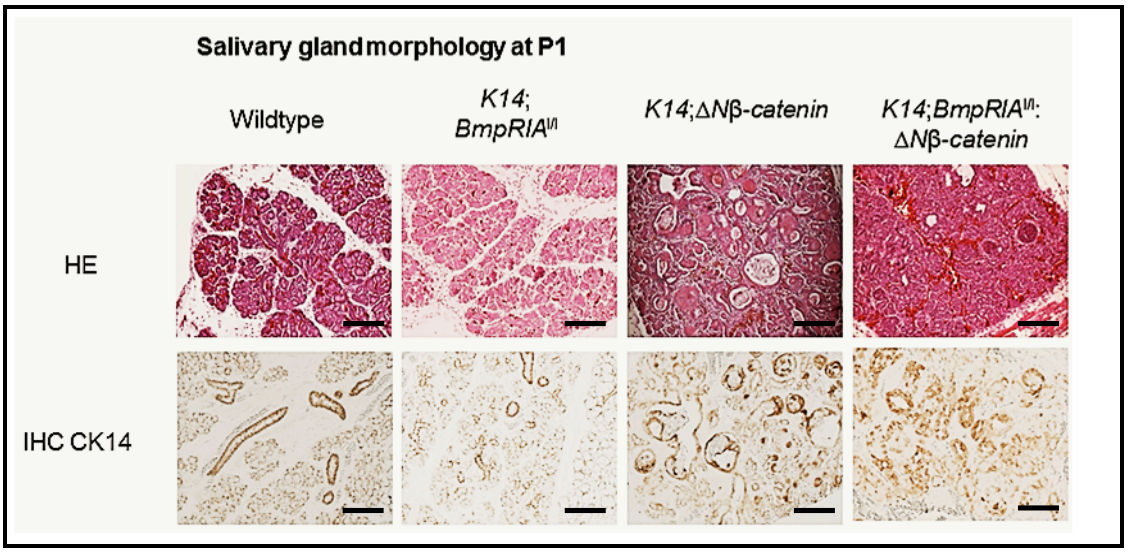
Major points:

1) There are several sub-types of salivary gland squamous cell carcinomas, such as the mucoepidermoid and adenoid cystic types, with different gene expression profiles (Gibbons et al., 2001, The Laryngoscope 111, 1373-1378). The authors should specify the sub-types of the human samples that were analyzed (showed in Suppl. Table 1), and the type of salivary gland SCCs that develop in the mouse.

Answer: Salivary gland cancer is indeed a heterogeneous tumor group, comprising a large number of tumor subtypes, such as adenocystic carcinoma (WHO histological classification ICD code 8200/3), mucoepidermoid carcinoma (ICD 8430/3) or squamous cell carcinoma (ICD 8070/3) (Barnes et al, 2005). The latter three subclasses represent independent malignant epithelial tumor subtypes that can be clearly separated from each other by pathologists. We apologize for any confusion that may have arisen from our manuscript, but we want to clarify that our study focuses exclusively on squamous cell carcinomas of the salivary gland in mouse and men. Our study does not include tumors with mucoepidermoid and adenoid characteristics, as verified by our collaborating pathologists. To the best of our knowledge and supported by the literature, squamous cell carcinomas by definition cannot be sub-classified in mucoepidermoid and adenoid cystic types, because all three represent different subtypes. Therefore, we cannot provide a further sub-classification of the squamous cell carcinomas used in our study. We now specify this fact in the legend of Supplementary Table 1.

2) An important question is whether the K14-Cre transgene used for these studies is expressed in all or only a subset of epithelial cells in the salivary gland (acinar, ductal and/or myoepithelial cells), as other K14 Cre transgenes have been previously reported to target only a portion of epithelial cells in this gland (Jonkers, Nature genetics 29, 418-425). When does K14-Cre start to be expressed in these various populations during development?

We agree with the reviewer that it is an important point to confirm the significance of the used genetic mouse model. We have used a Cre-reporter mouse line (LacZ) and in addition, examined the expression of Keratin 14 by *in situ* hybridization (Suppl. Fig. 1C-E). Our new data show that the used K14-Cre line is specifically active in the basal and myoepithelial ductal compartment of the salivary gland. Our K14-Cre line shows no activity in acinar cells. This is also consistent with our *in situ* hybridization data and in agreement with data published by *Jonkers et al. (Jonkers et al, 2001)*. We did not examine, at which time-points K14-Cre is activated in the different salivary gland cell populations during development. We have characterized the salivary glands of our mouse mutants at P1 by RNA microarray and histological analysis. Indeed, we found striking transcpriptional changes as well as profound changes in the salary gland morphologies of β-cat^{GOF} and double mutant mice as compared to WT mice (already at postnatal day P1). This does not rule out that the K14-Cre may be active at much earlier times, but it clearly argues that the K14-Cre is active in the salivary gland during perinatal development, in order to manifest such a phenotype. Below, we provide the reviewer with additional H&E stainings as well as Cytokeratin (CK) 14 immunohistochemistry (IHC; Bars 200 μm).

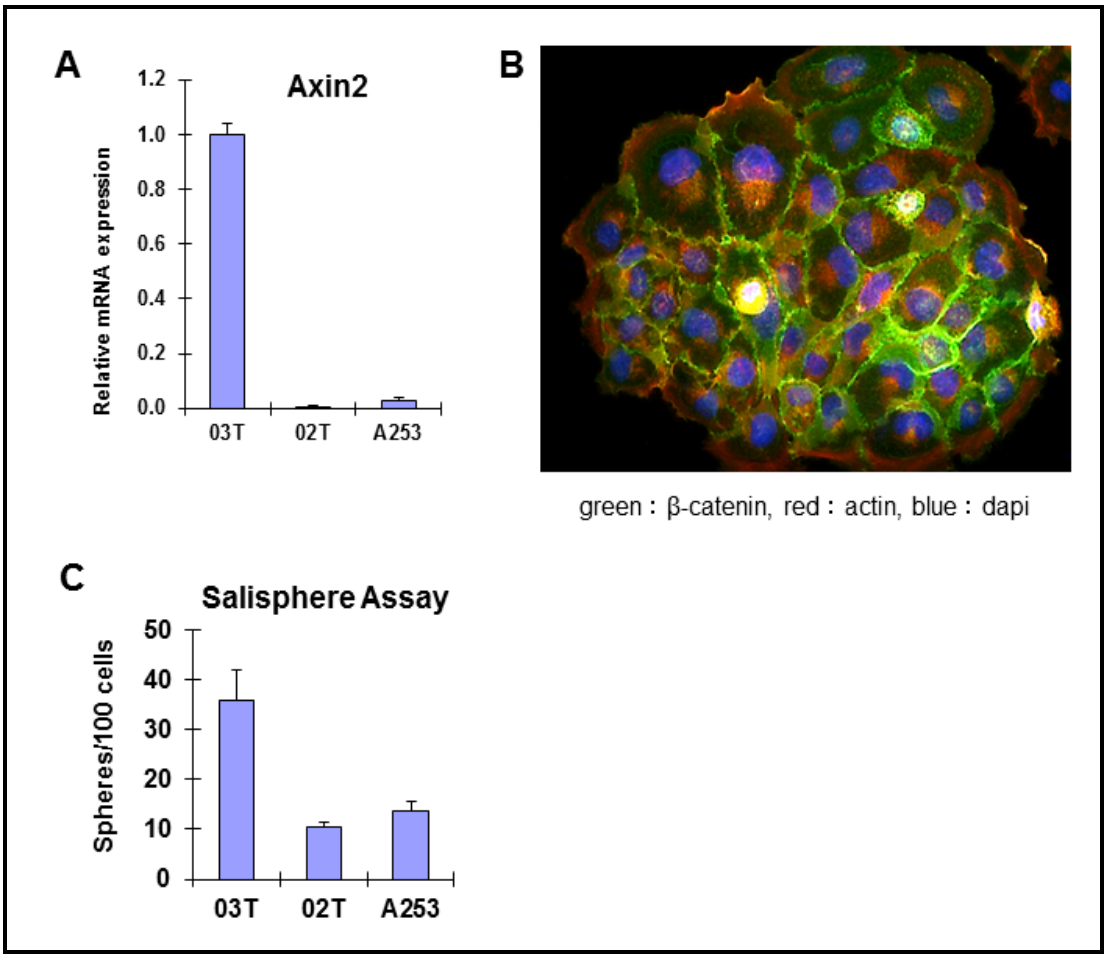


3) Along these lines, and in view of recent work, for instance by the Kopan's laboratory on skin SCCs, it is important to know whether all tumor cells are K14 Cre positive and have undergone the expected gene rearrangements.

A good point also: we have now addressed this question by examining recombined alleles of the Bmpr1a and β-catenin genes by PCR (Suppl. Fig. 1G). The data show that K14-Cre-mediated recombination of the Bmpr1a and β-catenin genes can be detected in both $CD24^{+}CD29^{+}$ and $CD24^{-}CD29^{-}$ cells isolated from the primary tumors, suggesting that the majority of tumor cells is K14-Cre positive and has undergone gene recombination

4) In the human relevance section (page 10), the authors used several human head and neck cancer cell lines rather than existing human salivary gland SCC lines, such as H292 or A-253, that, for the sake of consistency, should be included in the analysis.

We have characterized the A-253 cell line and can show that they only lowly express the Wnt target gene Axin 2 and have a predominantly membrane-bound β-catenin. Therefore, we classify these as tumor cells with low Wnt/β-catenin activity', akin to the 02T, but quite different from the 03T, which is a 'high Wnt' cell line. Consequently, the A-253 cells have a low capacity to form salispheres, similar to the 02T 'low Wnt' cell line. Since these cells behave so similar to the O2T cells, we have previously characterized, we would like to provide the reviewer with the data for the A-253 cell line without adding the data to the main manuscript (A; qt-PCR for the Wnt target gene Axin2, B; Immunofluorescence analysis for β-catenin, C; Salisphere formation capacity, please see also Supplementary Figure 7A for comparison of nuclear localization of β-catenin in the high Wnt cell line 03T). The second cell line mentioned by reviewer 1, H292 (NCI-H292, ATCC CRL-184; http://www.atcc.org/Products/All/CRL-1848.aspx), is considered to be a lung carcinoma cell line. We have not tested H292 cells, because the focus of our manuscript is on squamous cell carcinoma of the salivary gland.



5) It seems that in the double mutant mice, SCCs develop only in the submandibular gland. Did the authors also check the parotid glands? This is an important point, given the fact that, in human, most tumors arise in the parotid and not submandibular gland.

> We agree with the reviewer on this important point. Together with our pathologist, we have evaluated all main tissues from these mutant mice including all sub-structures from the salivary glands from P1 to P90 or until date of death. All tumors detected were within the submandibular salivary gland. We have now emphasized this point in the Result section on page 6: "After full necroscopy, a pathologist C.L. determined that these tumors exclusively arose from the submandibular salivary glands. The tumors were classified as SG-SCC by histopathological criteria, contained keratin pearls and expressed high levels of CK10 (Supplementary Fig. 2A, right, see also inset)."

Minor points:

1. Figure 1A: β-catenin staining seems to be mostly in spindle-like cells. Double staining with keratin antibodies is required to distinguish tumor from stromal cells.

> We have addressed this point by immunofluorescence analysis to test for co-localization of β-catenin and Cytokeratin 10 in tumor tissue (Supplementary Figure 1A). Our new data show that nuclear β-catenin can be found in both CK10-positive as well as CK10-negative cells in the bulk tumor and at the tumor front. This finding is consistent with the fact that these tumors show areas that are differentiated as well as areas with undifferentiated cells. This is rather typical of tumors that are Wnt/ β-catenin dependent where epithelial-to-

mesenchymal transition (EMT) can occur. In our tumor model, this leads only to localized expression of differentiation markers (as CK10). Spindle-like cells, mentioned by the reviewer, could also be a sign (byproduct?) for EMT of epithelial tumor cells. We mention these points in the new Figure legend.

2. In Figure 1B, the authors claim that up to 75% of aggressive SGSCC are nuclear β-catenin positive and pSmads negative. Representative images need to be shown. How many sections/fields have the authors examined? What was the positive control for pSmad staining?

We show representative images for SCC tumor cells with nuclear β-catenin and negative pSmad in the upper panel of Figure 1A. To support our quantification data, we now have summarized all b-catenin and pSmad staining results in Supplementary Table 1. Tumors were examined and quantified as follows: three observers (P.W., C.L., and U.Z) performed quantitative analysis of the tissue specimen without knowledge of specimen identification, and 3-5 sections from each tumor were evaluated. As a positive control for pSmad staining served the central, differentiated tumor areas (as shown in inset of Figure 1A and Supplementary Figure 2B) that showed ductal cells with nuclear pSmad. Moreover, our lab is using pSmad staining on a regular basis and has established a reliable quality control for such pSmad assays, e.g. pSmad staining in cardiac cells; see also Klaus *et al.* (Klaus et al, 2012; Klaus et al, 2007).

3. In Figure 3G, the CD24 and CK6 pattern of staining is very different in the sections from β-CatGOF versus β-CatGOF x Bmpr1aLOF mice. Images for the double mutant mice have much higher background levels and less green signal (β-catenin). Is it possible that the samples were not equally processed?

This is a valuable critique. Figure 3G was part of the regeneration experiment, we showed in the last version of the manuscript. Due to the reviewers concerns and to streamline our manuscript, we have now completely removed all data on regeneration including Figure 3G in our revised manuscript.

4. Figure 6 F,G: statistical significance of differences between samples plus/minus ICG needs to be assessed.

We have now included *p* values and statistical significance in the graphs of the ChIP experiment (former Figure 6F,G; now Figure 5A,B).

5. Supplemental Figure 1C: it looks as if hair follicles in the double mutant mice degenerate into cysts. Hence, how did the authors quantify hair follicle density?

We agree with the reviewer that the provided images had to be improved to better highlight increased hair follicle density or hair follicle numbers. The revised Figure now clearly shows accumulation of hair follicles in double mutant skin, when compared to wildtype controls (Supplementary Figure 2D).

6. Supplemental Figure 1E : K10 may not be a good marker for salivary gland cell differentiation, since, at least in normal mouse salivary glands, K10 is not expressed in acinar cells. Also, are K10 positive cells also K14 Cre positive?

We agree that CK10 is not an appropriate marker for normal salivary gland differentiation. CK10 can be used, however, as a valuable marker for squamous transformation of epithelial cells (Chu & Weiss, 2002). Figure 1C of the former manuscript version (now Supplementary Figure 4D) was intended to show that transplanted tumors consist of differentiated and undifferentiated tumor areas. Since primary salivary gland SCC of the double mutant mice expresses CK10, we have examined this marker. The new manuscript

text now better defines our rationale to use CK10 as a marker in tumor transplants. In the new version, we also provide evidence that CK10-positive cells co-express CK14 (Supplementary Figure 4E).

Referee #2:

This manuscript investigates the role of the Wnt pathway in driving cancer stem cell proliferation, using salivary gland tumors as a model.

The introduction is over concise. A couple of lines explaining where the story is going and linking sentences to connect the paragraphs would help readers follow the train of thought. A diagram explaining the anatomy of the salivary gland and the location of the different cell populations would greatly assist the general reader.

We thank the reviewer for these valuable critics and have made many changes in the manuscript for better text intelligibility and sentences connecting paragraphs. Moreover, we now also provide the requested diagram to illustrate the anatomy of a mouse salivary gland and further supplementary information (Supplementary Figure 1B-F).

The authors begin by examining expression of b catenin and nuclear smad1/5/8 in head and neck squamous cell carcinoma (SCC) arising from the salivary glands and other locations. Nuclear b-catenin is present in undifferentiated areas of the tumor and nuclear smad1/5/8 in differentiated areas, suggesting these staining patterns correlate with differentiation (Fig 1A). Are the images in Fig 1A serial sections of the same tumor (it is difficult to correlate the H and E with the immunohistochemical panels)? Where do the insets come from in the main panel? Scale bars are missing throughout Figure 1.

We now provide this information more precisely in the text and figure legends of the revised manuscript. The sections in Figure 1A are indeed serial sections, and the insets refer to central tumor parts that either show membrane-bound b-catenin or pSmad-positive nuclei of ductal cells. We say this now in the text/Figure legends. We also added scale bars throughout Figure 1.

The correlation with grade (the criteria used to score tumors as grade 2 and 3 need to be clearly explained for non-specialist readers) is consistent with the link between the marker expression and differentiation (Fig 1B).

We now provide explanations how tumors were graded as grade 2 and 3 in the text and Figure legends. These criteria were employed by our collaborating pathologists, who are specialists in head and neck tumors. We have relied on the tumor grading criteria that were defined in: World Health Organization Classification of Tumours, Pathology & Genetics of Head and Neck Tumours. IARC Press: Lyon 2005, which are now cited (Barnes et al, 2005).

Given the heterogeneity of SCCs, both within individual tumors and between lesions it is essential to specify exactly the scoring criteria used for the immunostaining.

We agree with the reviewer that salivary gland tumors in general and SCC in particular are heterogeneous tumors (please see also our response to major point 1 of reviewer 1). The revised manuscript now provides the scoring criteria used for the immunostaining

in the Material and Methods section as follows: Immunohistochemistry evaluation. Three observers (P.W., C.L., U.Z.) performed quantitative analysis of the tissue specimens without knowledge of specimen identification. Scoring was based on intensity and percentage of positively stained cells for β-catenin, pSmad1/5/8 and MLL1 by immunostaining as follows: i) for β-catenin; intracellular localization as analyzed for Fig. 1A: n, nuclear in ≥ 20% of the cells; m, membrane; cp, cytoplasmic. Nuclear score "n" as analyzed for Fig. 6F,G: low; ≤5%, medium; 5-25%, high; ≥25%), ii) for p-Smad 1/5/8; as analyzed for Fig. 1A: -/detectable in ≤ 10% of the cells; +/detectable in ≥ 10% of the cells, and iii) for MLL1; Nuclear score as analyzed for Fig. 6F,G: low; ≤5%, medium; 5-20%, high; ≥20%). Discrepancies were resolved by a second examination using a multihead microscope. Further scoring criteria for immunostaining and all staining results are now summarized in Suppl. Table 1.

Fig 1C shows nuclear b catenin expression in CD44 and CD24 positive cells. Given the weak evidence for CD24 and CD44 being stem cell markers in SCC it might be better to describe them as "candidate stem cell markers".

We now use the term "candidate markers for tumor propagating cells" for CD24 and CD44 in the text.

The scoring rules used to count percentages of double positive cells need to be given: are these percentages of all tumor cells or cells at the tumor front?

The given percentages refer to double positive cells of the entire tumors. We now provide a more precise description in the revised manuscript, in both text and figure legend for Figure 1.

The statement that "many" cells with nuclear b-catenin express CD24/CD44 is confusing, given the low proportion (3-6%) of double positive cells in figure 1B.

We have now revised the manuscript text and removed the description "many cells". We now state in the Results section on page 5: "A subset of nuclear β-catenin-positive cells from human SG-SCC and HN-SCC co-expressed the marker CD24 (Fig. 1A*,C, quantifications are shown for grade 2 and grade 3 tumors in B, percentages refer to all tumor cells) (Visvader and Lindeman 2008; Monroe et al. 2011) and the marker CD44, which is specific for tumor propagating cells in this human tumor type (Fig. 1C, right) (Prince et al. 2007; Visvader and Lindeman 2008)".

The evidence presented on human SCC does not support the claim that "High Wnt/b-catenin and attenuated Bmp signals lead to head and neck squamous cell carcinomas in humans".

We have modified the manuscript text to "High Wnt/β-catenin and attenuated Bmp signals correlate with high grade SCC" (first Results headline).

A genetic experiment in mice to investigate the effect of coexpression of a b-catenin gain-of-function mutant and null BMPr1a mutant in keratin14 positive cells is then described. It would be helpful to include a diagram summarizing these complex mutant animals and explain the pattern of Krt14 expression in the salivary gland.

We have addressed this point by providing a breeding scheme for our double mutant mice and CK14 expression patterns in the salivary gland (Supplementary Figure 1 B-G).

The image in Fig 1D is confusing as the lesion appears to arise from the mediastinum rather than the oral cavity.

In contrast to the location of human salivary glands, the main mouse salivary glands are located outside the oral cavity in the neck region where they are connected with a small isthmus (for an image, please see http://www.informatics.jax.org/cookbook/figures/figure45.shtml). The used double mutant mouse model develops salivary gland SCC in the submandibular gland exclusively. We have revised the manuscript text and provide this information to avoid confusion.

Supplementary Figure 1 lacks scale bars and images of control mice are needed in 1C to interpret the changes in the H and E panels.

We have revised this figure (former Supplementary Figure 1C, now Supplementary Figure 2 and 4D) and included scale bars and H&E staining from control mice. The epithelia of esophagus and forestomach from wildtype and double mutants shows only minor changes, and since it is not the major focus of the paper, we have withdrawn this statement in the revised manuscript. Nevertheless, the morphological changes in the skin and the accumulation of hair follicles in double mutants is dramatic (see new Supplementary Figure 2D).

The double mutant animals die from salivary gland lesions by 3 months of age, whereas the single mutants have no effect on survival over this interval. It would be helpful to confirm that the mouse tumors have similar changes in b-catenin and smad staining to those shown for the human tumors.

To satisfy this critique, we provide the stainings for β-catenin, and pSmad in the double mutant mouse tumors. Moreover, we confirm the high Wnt/β-catenin activity in these tumors using Axin2 in situ hybridizations in the salivary gland SCC of the double mutant mice (Supplementary Figure 2B). Please compare to the human tumor stainings in Figure 1A.

In the main text changes in apoptosis and gene expression shown in Fig S1B are said to be at p1, whereas the figure legend refers to p90.

We have now revised the text accordingly.

The authors then turn to analysis of CD24+ CD29+ cells. In Fig 2A it is essential to show isotype controls for each channel as the level of background staining may change significantly between normal and tumor samples.

We now provide the isotype controls in Supplementary Figure 3 A,B. Importantly, there are no changes for CD24, and small changes for CD29, which do not affect the double staining.

In the text it is stated that "In the tumors, CD24+CD29+ cells strongly clustered (Fig. 2B)". This is misleading. Fig 2B only shows Cd24 staining and even in tumors the proportion of double positive cells by FACS is under 10%. No evidence is provided that the double positive cells are clustered.

In order to avoid controversy, we have removed this statement from the text. It is true that Fig. 2B shows CD24 staining only. However, the morphological changes of double-mutant glands at P1 as well as the majority of immunoflouresence stainings performed, is consistent with the double positive stem cells being clustered.

Staining of cytospins argues that the double positive cells from tumors are proliferating and generate growing tumors. It would be helpful to know the proportion of cells which are not double

positive that express proliferation markers, to exclude the possibility that the markers simply enrich for proliferating cells rather than "cancer stem cells".

> We thank the reviewer for this critique and have analyzed freshly isolated tumor cells by FACS for triple-staining with CD24, CD29 and the proliferation marker Ki67. The new data show that high coexpression of the surface markers CD24 and CD29 did not exclusively enrich for proliferating cells. Also CD24 or CD29 negative tumor subpopulations can proliferate (Supplementary Figure 4A,B). For instance, P4 cells also strongly proliferate (40%), but these are not epithelial cells, since they are not high in CD24. These are stromal cells, as they express vimentin and fibronectin. They therefore cannot be tumor propagating cells; we have not examined them by transplantation (see corresponding text in Results)

The focus then shifts to the role of the double positive cells in regeneration. There is no mention of how this was done in the methods section. Axin2cre/reporter mice reveal an upregulation of wnt signalling in regenerating glands in the intercalating duct cells. The single mutant mice regenerate more rapidly than controls and have a higher proportion of double positive cells (Fig 3). Validation of CK6 as a proliferation marker in each tissue is required, eg by comparing the proportion of BrdU positive cells in the CK+ and - cell populations. It is not clear how this work fits with the cancer stem cell theme and the manuscript might be better without it.

> We thank the reviewer for the critique and suggestion and have removed the whole regeneration chapter from the revised manuscript.

The manuscript then returns to cancer stem cells. A third surface marker, SSEA1 is found to be co-expressed with CD24 and CD29. Array analysis and Wnt inhibition demonstrates the expression of a set of Wnt responsive developmentally significant genes in the double positive cell population (Fig 4). Global changes in histone marks are also evident comparing double positive cells from single and double mutant mice. Similar changes are seen in Human SCC cell lines in vitro. In sphere forming cultures from double mutant mice Wnt inhibition retards sphere growth, an effect blocked by HDAC inhibition (Fig 5). In Figure 6 a role for MLL1 is investigated.

Figure S5C is unclear...high power insets showing the channels separately are required.

> We have addressed this latter point and now provide high power insets showing the channels separately. We now show that MLL1 is associated with the tumor propagating cells in whole salivary glands. Nuclear Mll1 correlates with the rare $CD24^{+}$ cells (Supplementary Fig. 7E).

Interestingly, Mll1 or b-catenin knockdown promoted differentiation, but these experiments would be strengthen by rescue of the phenotype with the relevant siRNA resistant constructs.

> Yes, a biological rescue experiment can sometimes be revealing. We have extensively used ICG-001 as means to counteract β-catenin action in the double-mutant tumor propagating cells. To assess the specificity of this drug, si-β-catenin experiments were performed. To circumvent any issues regarding the specificity of the si-probes, we used a pool of four individual sequences (SMARTpool, Dharmacon). We have used this approach repeatedly, e.g. (Fritzmann et al, 2009). Given, that our manuscript was received as rather bulky ("*large quantity of data")*, we believe that this additional experiment is outside the scope of the present manuscript.
> For MLL1, we have attempted to provide additional genetic evidence. MLL1 is a rather large protein (Cosgrove & Patel, 2010), which is proteolytically cleaved into two peptides. We failed to express the entire full-length MLL1 (3063 amino acids!) by retrovirus. Even the 300kDal N-terminus alone, could only be expressed about 1.5 fold. To create a si-resistant MLL1 that allows an efficient overexpression is beyond our current experimental possibilities.

Overall, there is a large quantity of data here, but the manuscript is poorly structured and hard for a non-specialist to follow.

We thank the reviewer for this critique and have now removed data (about regeneration), structured the manuscript differently, provided more introduction and explanatory schemes for improved readability and intelligibility of the text.

There are also some significant problems in the interpretation of the results.

We have addressed the critiques raised by the reviewer. Wherever possible, we have added new data (such as work on additional human cancer cell lines, additional MLL1 immunofluorescence staining in human tumors, improved the representation of various staining and quantifications). In order to avoid conflicts regarding the nature of the double positive tumor cells, we first toned down some of the conclusions drawn ("stem cells") and also provided additional text to explain this fact. Thus, we believe to have alleviated the problems regarding the interpretations of the results.

This work could be substantially improved by adding additional explanation for the majority of readers who are unfamiliar with the salivary gland, removing material not directly relevant to the cancer stem cell theme *and addressing the specific points above.*

Again, as stated before, we have removed the regeneration chapter and focused entirely on the cancer cell subject, and have addressed the points made by the reviewers (see also above).

Referee #3:

Wend and colleagues generate a double transgenic mouse model carrying a gain of function in beta-catenin and loss of function in Bmpr1a. These mice develop salivary gland tumors. The authors then isolate CD24/CD29 positive cell populations and show evidence that this population carries the cell of origin of the salivary tumors. Using a Wnt/beta-catenin inhibitor ICG-100 the authors can force these cancer cells towards differentiation. As a mechanism the authors suggest that Wnt/beta-catenin cause transcriptional permissive chromatin via MLL1.

The manuscript is dense and contains a tremendous amount of data. However, the rationale for the experiments is not always clear and it is difficult to understand what the original hypothesis of this work was and what precise conclusions can be drawn from the experiments.

We thank the reviewer for these critics and have made major changes in the manuscript for better text intelligibility. We focus entirely on the tumor aspects, removing the regeneration experiments, and make our hypotheses and the conclusions drawn more clearly. We have added introductory and explanatory conclusions to many chapters.

My main concern is that the paper does not show what the title suggests. (1) There is no direct evidence provided that Wnt/beta-catenin drives MLL1 activity in salivary glands tumors. (2) The actual evidence that the isolated CD24+/CD29+ tumor population is indeed the cancer stem cell population of this tumor is not sufficiently substantiated. All that is shown is that the CD24+/CD29+ tumor population can produce tumors in a transplantation experiment. I believe these claims either need to be supported by more data or the authors must tone down their claims throughout the manuscript.

We have now addressed these concerns and toned down our claims in the manuscript text.

(1) The major concern that MLL1 is not driven by Wnt/beta-catenin likely hinges on the fact, that we do not yet know how precisely beta-catenin and MLL1 co-depend. In the simplest version, beta-catenin activates the MLL1 gene (see RT-PCR in Fig. 3C; ChIP in Fig. 5), which then performs Wnt/beta-catenin dependent or independent actions. A more sophisticated scenario is that the beta-catenin and MLL1 proteins cooperate. What implication does this scenario entail? First, both proteins interact (for which you'll find authorative precedents). Second, that their interaction or co-operation leads to the epigenetic changes we have observed. Although we can't make statements about the interaction of the two proteins, we show persuasive evidences that the Wnt/beta-catenin-dependent epigenetic changes are absent, if we diminish either beta-catenin (activity or expression) or MLL1 (expression). Moreover, we focused on the fact that even human SG tumors show high MLL1. Indeed, nuclear MLL1 staining is a productive tool to discriminate the most aggressive subtypes of human cancers (Fig. 6F-G). Hence, we rephrased the title "*Wnt/β-catenin drives MLL1 activity in salivary glands tumors"* into "*Wnt/β-catenin signaling induces MLL1 to create epigenetic changes in salivary glands tumors"*.

(2) We have toned down our conclusion about the transplantation experiments with $CD24^+CD29^+$ double-mutant tumor cells. We now use the term "tumor-propagating cells", which has been used in very similar experimental setups and recently also in the EMBO Journal (Lapouge et al, 2012). We have added more data on the transplantation experiments and improved the presentation of our results (Fig. 2F,G and Suppl. Fig. 4E-F).

However, the authors convincingly show that activation of beta-catenin and inhibition of Bmp signaling can cause salivary tumors in mice. These tumors contain a highly tumorigenic cell population (CD24+/CD29+), which can be forced towards differentiation using a Wnt/beta-catenin inhibitor. This part is interesting and in my opinion gets lost in the current version of the manuscript.

We have now rearranged the manuscript and made the tumor aspect the main focus. We believe that the differentiation aspect is now a very central portion of the manuscript.

1. What is the relevance of beta-catenin activation and deletion of BMP signaling within the CD24/CD29 positive tumor cell population in mouse salivary tumors? The authors do not provide evidence for high nuclear beta-catenin and absence of phospho Smads in this population.

We agree with the reviewer that it is an important point to prove the relevance of high Wnt/b-catenin and low Bmp signaling in the $CD24^+CD29^+$ tumor cell population. We addressed this question and provide now evidence that high $CD24^+CD29^+$ expressing tumor cells also exhibit high levels of nuclear b-catenin. Moreover, these cells also co-express SSEA-1, as shown in Figure 3A,B. The chosen FACS strategy uses three markers but can confirm high nuclear b-catenin in high $CD24^+CD29^+$ cells. In addition, our revised manuscript now includes analysis of cytospins from $CD24^+CD29^+$ tumor cells for phospho-Smad1/5/8, confirming low Bmp signaling in this population, when compared to the entire tumor (Suppl. Fig. 5A,B). Overall, we have now found a significant correlation between high nuclear β-catenin and low pSmad in mouse and human tumor cells at the *in vivo* and *in vitro* level.

Is the increase in proliferation within this population directly due to beta-catenin activation and inhibition BMP signaling or is it indirect?

Our microarray and siRNA experiments and inhibitor treatments using the β-catenin inhibitor ICG-001 with $CD24^+CD29^+$ tumor cells clearly show that Wnt/β-catenin signaling is supporting growth and self-renewal of the tumor cells and tumor

propagating cells. Our array data also provide evidence that Bmp signaling is important for the induction of apoptosis in β-cat^{GOF} single mutant mice, and if lost in the double mutant mice, proliferation is further increased.

2. Figure 1: The rationale for testing nuclear beta-catenin co-localization with CD44 and CD24 in the context of the manuscript is unclear. The authors name them as stem cell markers and it is unclear onto what observations or publications the authors base this assumption with respect to squamous cell carcinomas or salivary glands.

The rationale for this experiment was to show that cells with high Wnt/β-catenin signaling express known stem cell markers, such as CD24 and CD44. CD44 has been shown to be a more general cancer stem cell marker, but also in head and neck cancer (Prince et al, 2007). However, the correlation with nuclear β-catenin has not been shown in detail yet. We have tested the expression of CD24 and the correlation with nuclear β-catenin, because CD24 is a known stem cell marker in the mouse system, and we asked the question how well the expression of these markers is conserved between mouse and men with regard to cells with nuclear β-catenin. Reliable and robust stem cell markers for salivary glands that also could serve as markers for tumor propagating cells or cancer stem cells have not been established so far. We attempted to use this marker combination, amongst others to enrich for tumor propagating cells in the salivary gland SCC of our double mutant mice.

3. Along the same line; one would probably expect a co-localization of nuclear beta-catenin with CD44 in the invading front of the tumor but the IF shown in figure 1C does not seem to present that.

We agree with the reviewer that nuclear b-catenin and CD44 could be expected at the tumor fronts, because these are tumor areas with high nuclear b-catenin signaling, and CD44 has been shown to be a Wnt/b-catenin signaling target gene. Indeed, we can detect those cells at the tumor front. However, CD44 was reported to be almost ubiquitously expressed in the standard form of CD44 (CD44s) and also for alternative splice variants (e.g. CD44v6) in HNSCC and in most normal epithelia of the head and neck area (Gires, 2011; Kawano et al, 2004). In our staining, there are areas with nuclear b-catenin in the tumor that are CD44-positive and those areas were represented in the shown image (Fig. 1C).

Furthermore, the number CD44/beta catenin co-localizing cells are not quantified and the authors do not state if there are any triple positive (beta catenin/CD24/CD44).

We have added a quantification of CD44$^+$/nuclear b-catenin cells in the manuscript (Fig. 1C, lower right of right panel). We have not tested triple staining on tumor sections, however, we can show that high Wnt cell lines co-express also high amounts of CD24/CD44 (Supplementary Figure 6C). Importantly, co-expression of these markers can be induced by CHIR in low-Wnt human tumor cell lines.

4. Why do the authors then chose to flow sort CD24+/CD29+ cell populations from the mouse tumors?

CD24 and CD29 are well-characterized stem cell markers in a number of other organs of mice, for instance the mammary gland, but also in other tissues (Huelsken et al, 2001; Jiang et al, 2011; Lawson & Witte, 2007; Panchision et al, 2007; Sagrinati et al, 2006; Shackleton et al, 2006; Vermeulen et al, 2008). Further supporting evidence for the CD24/CD29 marker combination was recently provided by Nanduri et al., who demonstrated that salivary gland function could be alleviated after CD24$^+$CD29$^+$ stem cell transplantation (Nanduri et al, 2011). Several stem cell markers were used for the isolation of salivary gland (stem) cells, such as c-kit, CD133, CD49f, and the combination of CD24/CD29. CD24$^+$CD29$^+$ cells were superior to the others in their capacity to reconstitute irradiated salivary glands following transplantation (Nanduri et al, 2011). Since mice are

capable to survive the removal of mammary glands, a rare in vivo reconstitution assay was made with mammary gland stem cells (Shackleton et al, 2006). These technically highly challenging experiments can't be performed with salivary glands, since mice are not capable to survive the removal of this organ. We have examined the differentiation of $CD24^+CD29^+$ cancer stem cells functionally *in vitro* by analysis of marker expression and electron microscopy (Supplementary Figure 6F,G). Differentiation was possible, if cells were reprogrammed by either siβ-catenin, siMLL1 or ICG-001 treatment (Figures 4C-E and 6E). These findings provide strong arguments that despite their tumorigenic nature, these cells retain an enormous capacity for stemness (i.e., not only to self-renew, but also to be able to differentiate). This is entirely consistent with the findings of Nanduri *et al.*, who have also linked $CD24^+CD29^+$ markers with stemness and regenerative capacity (Nanduri et al, 2011).

And the statement that CD24+/CD29+ strongly clustered (Fig. 2B) is not reflected in the figure. CD29 staining is not shown and one would expect a general basement membrane staining.

We agree with the reviewer and have removed this statement from the manuscript.

5. For both mouse and human tumors the authors should show where the Bmpr1a is actually expressed and whether it overlaps with CD44, 24 or 29.

We have attempted to answer this question using different antibodies against Bmpr1a. However, none of the tested antibodies gave convincing results in immunohistochemistry or immunofluorescence experiments.

6. Figures 2F and G are unusual presentations of transplantation assays and should rather include the information of how many times the experiments have been performed and how often those resulted in the formation of a tumor.

We have revised the tables that summarize the transplantation studies. They now show how many times the experiments have been performed and how often tumor formation could be detected (Figure 2F,G, Suppl. Fig. 4F,G). More data highlighting the serial transplantation assay are now provided in Suppl. Fig. 4F,G.

7. Serial transplantation assay of the isolated cancer cell population is currently one of the gold standards to define a cancer stem cells population. The authors claim that the CD24+/CD29+ population gave rise in serial transplantation assays but do not provide the data.

We agree with the reviewer that the data on serial transplantation assays have to be provided and therefore, we have included the results in the revised manuscript (Suppl. Fig. 4F,G).

8. The rationale for the wounding experiments is unclear. Just because the CD24/CD29 population can contribute to tissue regeneration does not qualify the cells as stem cells. It is also unclear onto what experimental results the authors build their statement that 'single mutant tissues regenerated faster'. The authors refer to figure 3C, which only shows that the number of K14+ proliferating cells increased. Whether normal tissue architecture has been achieved is not shown.

We agree with the reviewer that data on regeneration would need much more in depth analysis and followed the suggestion of the reviewers to remove the regeneration chapter from the revised MS.

9. The overall increase in H3K4 trimethylation can be due to increased proliferation of an undifferentiated cell population but is not solely or uniquely altered in cancer stem cells.

We thank the reviewer for this important critique and agree that H3K4me3 is not only a characteristic of high $CD24^{+}CD29^{+}$ tumor propagating cells, but may also be detected in proliferating, undifferentiated cells. However, the cytospin and the more specific ChIP experiments clearly show that the tumor propagating cell population has a strong increase in H3K4me3, and that this histone mark has also functional relevance since either si-β-catenin or ICG-001 leads to decreased H3K4me3 levels resulting in attenuated proliferation and induction of differentiation. It is also important to remember, that there are CD24/CD29 single or double negative cells, which are proliferative (Suppl. Fig. 4A,B). However these cells do not show these high levels of H3K4me3.

10. Figure 5A; The authors test the effect of the beta-catenin inhibitor on cell proliferation of double mutant cells. The effect of the drug might be simply explained by negatively regulating cell proliferation. It would be interesting to see how cell proliferation is affected in the single mutant cells.

We have attempted to address this question in detail. However, this experiment hinges on the propagation of wt or single mutant cells. So far, we were unable to culture wt or $CD24^{+}CD29^{+}$ single mutant cells *in vitro*.

11. What happens to cellular localization of Mll1 when the cells are treated with CHIR?

We have addressed this question and have treated the tumor propagating $CD24^{+}CD29^{+}$ cells with CHIR, which lead to higher concentrations of nuclear Mll1. This result can be explained by the fact that in the double mutant mice still one allele of wildtype b-catenin is functional. The wildtype protein product is indeed affected by the CHIR-induced inhibition of GSK3b leading to translocation of b-catenin to the nucleus. This effect can be seen in Figure 6A. Active Wnt/b-catenin signaling can induce the expression of *Mll1* followed by a translocation of the protein to the nucleus of the highly proliferating tumor propagating cells. This effect is now shown in Figure 6B.

12. The strong conclusion in the last paragraph are not supported by experiments: "We suggest that a beta-catenin / CBP / MLL1 complex drives self-renewal...".

As explained above, such a statement is beyond the scope of this manuscript. We agree with the reviewer and have removed our statement about the complex formation from the revised manuscript.

The authors only show in figure 6F and G that the overall level of H3K4 trimethylation in the promoters is lower when cells are treated with ICG-001. However, the cells differentiate in response to the drug and the reduced trimethylation levels might be the result of the increased contribution of the differentiated population, which has silenced the promoters. In other words, from the provided experiments one cannot distinguish between cause and consequence of H3K4 methylation changes at these sites.

In our revised manuscript, this point is now addressed with a time-course experiment, where we have treated tumor propagating cells with the Wnt/β-catenin inhibitor ICG-001. Subsequently, we have analyzed H3K4me3 levels by Western blot analysis followed by qPCR to probe the expression of the salivary gland differentiation gene Amylase and other genes from our stem cell-associated gene signature. The data show that H3K4me3 downregulation can be detected 12h after ICG-001 treatment, whereas changes in gene expression occur later (24h after ICG-001 treatment, Fig. 5C,D). These data suggest that the epigenetic changes precede the gene expression changes (e.g. Amylase and other stem cell-associated signature genes). We believe that this is a strong argument that we are able

to distinguish between cause and consequence, i.e. it is the β-catenin-dependent epigenetic changes (of H3K4me3) which causes the induction of differentiation genes leading to cellular differentiation and not the consequence.

Minor comments:

1. The manuscript would benefit from a more precise description of the figures, i.e. 'many nuclear beta-catenin-positive cells....(Fig. 1A-C)' does not seem to be reflected in the quantification when only less than 7% are double positive.

We have now revised the manuscript text. We now state in the Results section on page 5: "A subset of nuclear β-catenin-positive cells from human SGSCC and HNSCC co-expressed the marker CD24 (Fig. 1A*,C, quantifications are shown for grade 2 and grade 3 tumors in B, percentages refer to all tumor cells) (Visvader and Lindeman 2008; Monroe et al. 2011) and the marker CD44, which is specific for tumor propagating cells in this human tumor type (Fig. 1C, right) (Prince et al. 2007; Visvader and Lindeman 2008)".

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2nd Editorial Decision 22 April 2013

Thank you for your patience while we re-reviewed your revised manuscript. I am happy to inform you that in light of the comments from the original referees (provided below), we are ready to proceed with acceptance of the paper, pending modification of a few additional points.

- Referee #3 remarks that in order to demonstrate conclusively that the genes analyzed in Figure 3 mediate functions specific to the CD24/29 positive population from double knock-out tumors, the validation experiment should have included CD24/29 positive cells from single mutant mice. Although this point was not raised in the original review, it addresses an important issue. Therefore, I was wondering if you performed these control experiments and might have the requested data at hand for inclusion in the manuscript. If the suggested amendments cause major difficulties, I am very happy to discuss this further. In this case, please contact me by phone or e-mail according to your preference.

- Please be sure to include information regarding the number of biological replicates and the statistical tests used to create error bars for all Figure panels.

- Please add an author contribution statement.

- Additionally, we encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Therefore, I would like to invite you to provide a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed scans of all gel/blot panels used in the respective figures. These should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. A ZIP archive containing these individual files can be uploaded upon resubmission (selecting "Figure Source Data" as object type) and would be published online with the article as a supplementary "Source Data" file.

- Finally, please complete and sign the linked license agreements (see below).

I will now return your manuscript to you for one additional round of minor revision. After that we should be able to swiftly proceed with formal acceptance and production of the manuscript!

If you have any questions, please do not hesitate to contact me directly.

REFEREE COMMENTS

Referee #1

The authors have adequately addressed all my concerns.

Referee #2

The authors have significantly improved the text and addressed the key points made in the first review. The manuscript is now suitable for publication.

Referee #3

In their revised manuscript Wend et al. carefully addressed all initial concerns, strengthened the data and clarified the rationale for each experiment. The manuscript is now well structured and focused. In fact, there are only very minor points that came to my attention:

Minor points:
1. The least sentence of the abstract concludes: "it suggests new strategies for therapy of solid tumor" . What do the authors mean in that case? ICG-001 is already described for being highly likely to enter clinical phase I trials in the near future.
2. Aim of figure 3 is "to elucidate the mechanism that potentiate the self-renewal of tumor propagating cells". The authors identify a couple of genes over-expressed in the double knockout versus the single tumors. However, the validation is solely performed in double knockout cells. If the authors want to demonstrate that these RNAs mediate functions specific to the CD24/29 positive population from double knockout tumors, the experiments should have been done alongside CD24/29 positive cells from single mutant mice.
3. The text should be standardized for Mll1 or MLL1, or the authors should entirely switch to the official gene symbol (mouse: Mll1; human: MLL).

Additional correspondence (author) 25 April 2013

Thank you very much for your email and your ongoing interest in our work. We are very happy and truly excited that you are now ready to proceed with the acceptance of our paper, pending modification of a few additional points.

With regard to Referee #3 and the remarks on the gene signature of Figure 3:

We agree that it would be helpful to include the analysis of CD24/29 positive cells from single mutant mice to validate the specific function of RNAs, which were examined in double-mutant CD24/29 positive cells. However, these experiments would require an in vitro culture of freshly isolated CD24/29 positive cells from single mutant mice for at least 3-4 days, to treat them with siRNAs or ICG-001. As mentioned in the manuscript (pg. 12) and in our point-by-point letter (answer #10 to Reviewer 3), unfortunately, we cannot culture CD24/29 positive cells from single mutant mice in vitro, although we have examined a variety of cell culture conditions. The only cells that we could propagate in vitro are CD24/29 positive cells from double mutant mice, which were used for the analysis depicted in Figure 3C. This alone was very difficult, until we discovered the additional requirement of Hepatocyte Growth Factor. Therefore, we regret to say that a functional in vitro analysis of CD24/29 positive cells from single mutant mice is beyond our current experimental capabilities. Nevertheless, we can provide an mRNA expression analysis (qPCR) of the gene signature for freshly isolated and untreated CD24/29 positive cells from single and double mutant mice. This validation experiment has been performed to verify our microarray data. If you believe that such data are helpful to strengthen the manuscript and to address this point, we will be happy to include these results.

In the required additional revision of our manuscript, we will address all other points raised by Reviewer 3 and, as requested by you, we will include information on biological replicates, statistical tests, author contributions and will provide all necessary source data.

We hope you can agree with our arguments, and we are looking forward to receive your reply, so that we can promptly resubmit our revised manuscript.

Additional correspondence (editor) 25 April 2013

Thank you for your comments in response to my decision. Since the additional experiment proposed by reviewer #3 is technically not feasible, the manuscript can be accepted without this data. I would suggest to include the qPCR analysis of the gene signature performed in freshly isolated cells from single and double mutant mice to validate the microarray data.

I am looking forward to your resubmission!

2nd Revision - authors' response 07 May 2013

We have addressed the residual points by reviewer 3 as follows:

1. The least sentence of the abstract concludes: "it suggests new strategies for therapy of solid tumor". What do the authors mean in that case? ICG-001 is already described for being highly likely to enter clinical phase I trials in the near future.

> We have modified this sentence. It now reads “Further, it supports new strategies for the therapy of solid tumors.”

2. Aim of figure 3 is "to elucidate the mechanism that potentiates the self-renewal of tumor propagating cells". The authors identify a couple of genes over-expressed in the double knockout versus the single tumors. However, the validation is solely performed in double knockout cells. If the authors want to demonstrate that these RNAs mediate functions specific to the CD24/29 positive population from double knockout tumors, the experiments should have been done alongside CD24/29 positive cells from single mutant mice.

> As explained in our email correspondence with you on April 25, 2013, a functional *in vitro* analysis of CD24/29 positive cells from single mutant mice is beyond our current experimental capabilities. We thank you very much for the decision that the manuscript can be accepted without this data, as mentioned in your email reply of April 25, 2013. In the revised manuscript, we have now included the requested qPCR analysis and a new Figure

to confirm the tumor propagating cell gene signature (Supplementary Figure 5C). The analysis was performed with freshly isolated CD24/29-positive cells from single and double mutant mice to validate the microarray data.

3. The text should be standardized for Mll1 or MLL1, or the authors should entirely switch to the official gene symbol (mouse: Mll1; human: MLL).

We have followed the reviewer suggestion to standardize the text and now solely use Mll for mouse and MLL for human. We believe this ensures a better legibility and intelligibility of the manuscript and is in accordance with the employed nomenclature in recent publications on MLL (Drynan et al, 2005; Gupta et al, 2010; Krivtsov & Armstrong, 2007; Mills, 2010; Yu et al, 1995).

As requested by you, the revised manuscript now also includes information on biological replicates, statistical tests, and author contributions and provides all necessary source data.

