THE EMBO JOURNAL

Manuscript EMBO-2010-74074

NANOG is essential in human glioblastoma acting in a cross-functional network with GLI1 and p53

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Review timeline:

Submission date:	02 March 2010
Editorial Decision:	23 March 2010
Revision received:	17 May 2010
Additional Correspondence:	01 June 2010
Accepted:	02 June 2010

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 23 March 2010

Thank you very much for submitting your research manuscript for consideration to The EMBO Journal editorial office.
As you will see from the comments enclosed below, all expert referees appreciate the potential insight provided by your study that proposes Nanog to drive tumorigenicity of glioma cells in a regulatory network with Hh/Gli1 and p53. However, careful reading of the reports from all referees indicates that at least the data provided in this version do not provide sufficient support to substantiate these claims. This seems partially a presentation issue but also due to the absence of key-experiments to convincingly establish causality. Major concern is expression and kind of distinct Nanog transcripts and the assignment of their individual contribution to gliomagenesis. It is also noticed that some of the functional assays seem redundant, which makes the paper extremely long without adding to the core message. In contrast, the proposed regulatory (and highly interesting) signaling networks are not sufficiently substantiated and need further thorough experimental testing. Despite these current obvious limitations and given the current interest in the topic, we decided to offer you the chance to convince the referees from the significance of your findings during a single round of major revisions. I urge you to take their comments serious and focus on the essential issues that are clearly documented in their comments, together with a necessary re-write of the that highlights the major advance presented in the study and to make it accessible also for non-expert readers. I do understand that this involves serious additional experimental work with partial uncertain outcomes. I would thus understand if you were to take the paper in its current form elsewhere to avoid later disappointments here. In case you were to pursue revisions for The EMBO Journal, I also have to remind you that it is EMBO_J policy to allow a single round of major revisions only. Thus, the final decision on acceptance or rejection entirely depends on the content and strength of the final version of your manuscript!

Yours sincerely,

REFEREE REPORTS

Referee #1 (Remarks to the Author):

In this manuscript, Zbinden et al first show the importance of Nanog in gliomasphere formation and tumorigenic potential of glioma cells. Then the authors provide evidence for cross talks among Nanog, Gli1, and p53. Overall, this is an interesting study that helps advance our understanding of signaling mechanisms in glioma stem cells. However, the paper suffers several major weaknesses.

MAJOR POINTS:

1) Although the loss-of-function data are strong, the inability to differentiate between Nanog1 vs. NanogP8 does not allow determination of their relative contributions to gliomagenesis. It is also not apparent whether the two NANOG1 'alleles' are valid alleles.
2) NANOGP8 is expressed (apparently) several fold higher than NANOG1 at the RNA level and further, the NANOG1-RFP reporter is not sufficient to track all clonogenic GBM cells. Since NANOGP8 promoter also exhibits putative GLI1 cis-elements, authors should focus their main efforts on the contribution from this locus.
3) The usefulness of the NANOG-RFP reporter is questionable. Clearly, a variant of NANOG mRNA is present in both subsets of RFP+/FRP- cells and both exhibit similar clonogenic potential. Are NANOG-RFP cells enriched in CD133? Inverse correlation with differentiation markers? Without further information this data is dispensable. The RT-PCR results in Fig. 1F are difficult to interpret.
4) Detection of endogenous NANOG protein in GBM is necessary to convince readers of the authors' findings. Along the same line, the inability to analyze NANOG protein leaves the question open whether shp53 and/or shPTCH1, which upregulate NANOG at the RNA level, also regulate NANOG protein.
5) There is no direct evidence that Nanog, Gli, and p53 cross-regulate one another.

Minor points:

1) The reported locations of putative Gli-binding sites seem to be inaccurate.
2) qPCR quantification and the y-axis NANOG mRNA levels (relative to?) need be clarified.
3) The manuscript has many typographical errors. Reference 'Varnat et al., 2009' was not cited. Legends generally lack sufficient experimental detail.

Referee #2 (Remarks to the Author):

This paper describes a new mechanism by which Nanog controls growth in glial tumours. Nanog is expressed only in a small subset of glioma cells in vitro, but the authors show that these cells are probably relevant for the promotion of tumour growth by maintenance of stemness.
The authors then show how the suppression of Nanog expression essentially halts growth of the tumours, by eliminating Nanog suppressed cells along several passages. The authors further show that p53 suppression also leads to a suppression of Nanog expression and henceforth a growth reduction.
The experimental data are sound, and supported by a number of good quality fluorescence images and other data. The conclusion is supported by their data.
That said, I was surprised by the unusual number of syntax, nomenclature and grammatical errors in this manuscript, plus occasional typos in text and figures. I really had a hard time reading it and the good quality of the data is obscured by the poor writing style. There is a lack of good structure in the individual chapters, in particular the results section contains sentences that are almost incomprehensible.

Major points:
The authors use glioblastoma cells/ cell lines, which are already published, but the reference (Clement 2007) contains only rudimentary molecular data of these glial tumours. The authors should

provide molecular details of these gliomas, in particular in the light of their variable growth, their claim of all GBM having a p53 mutation etc. Since the publication of the Clement's study in 2007, the IDH-1 and IDH-2 genes were discovered as being causative in the pathogenesis of most low grade gliomas, and consequently are also mutated in secondary GBM, in contrast to primary GBM, which typically do not have this mutation. In particular the moderate discrepancies within the GBM group (GBM1 and 10 vs. GBM-8 and GBM10-17) and compared to the Astrocytomas / Oligodendroglioma group may be correlated to the IDH mutation status.
The authors should also document the p53 status (mutation, LOH17p) of all their tumours and the 1p19q status at least of the oligodendroglial tumours.

Minor points:

- P7: did the authors examine this "equilibration" of Nanog positive and negative cells? Our experience with CD133+ and CD133- MACS sorted cells showed that enriched or depleted populations return to equilibrium when passaged in vitro. Did the authors observe a similar effect (with CD133+, or with NANOG+ cells)?
- Figure 1D: The legend refers to GBM-8, but the figure shows GBM12 in addition. Please clarify.
- Figure 2B, D, F: How many spheres were counted to generate these data?
- P5: "Analysis of NANOG protein were [sic!] hampered by the low levels of expression"; do the authors in fact mean that the cells express Nanog at low levels or instead that Nanog is expressed in a small number of cells? Their IF in Fig 1C suggests reasonably good expression in a small number of cells. Clarify, please
- P5 and Fig 1D: what is the profile of negatively sorted (CD133-) cells? Do they show a reduced Nanog expression? Di the authors carry out a FACS analysis with CD133 and their Nanog reporter? If they did, it may be interesting to show these data, to see the proportion of the following fractions: [CD133+, Nanog+]; [CD133-, Nanog-], [CD133+, Nanog-] and [CD133-, Nanog+].

A) Nomenclature

- : As a matter principle, the grade of all tumours should include the grading scheme, in this case probably the WHO grading scheme (e.g. Astrocytomas, WHO Grade III)
- The term Astrocytoma does not exist according to the WHO classification and should not be used.
- The expression "lower grade gliomas (astrocytomas and oligodendrogliomas grade II and III)" is misleading. Although used here comparatively to GBM, it also wrongly suggests that WHO Grade III is a low grade, which is factually incorrect. WHO Grade III Astrocytomas and oligodendrogliomas are high grade by definition. The results section should mention the number of tumours assessed. The medulloblastoma can be omitted, as only one sample was used.
- The first use of the term "GBM-8 cells" without introducing the nomenclature is confusing to the reader unfamiliar with the experimental setup. The author should ensure the term GBM -8 is consistently used (not GMB-8).
- Fig. 1A check AGII-1 (should read AII-1)

B) Syntax, Grammar:

- General: The results section is poorly written. Non-exhaustive examples are given below:
- P6, 1st para: As expected,...
- P6, para2: This result ... (this sentence is incomprehensible); larger gliomaspheres, ... larger in adherent cultures ...
- P7: Special location (?) specific?
- P8: para3: Difficult syntax
- P9: What do the authors mean with "challenged"?
- P9: not clear what "same tumours in the same mice" means.
- P10 "NANOG is essential for GBM's": essential for propagation of GBM tumour cells..
- P10: consistent(ly) diminished expression (adverb)
- P15: "endow GBM stem cells with their multiforme character: all astrocytic can have a variable phenotype, not only the GBM. The GBM is no more "multiforme" than different types of WHO grade II astrocytomas!

- General: Analysis (not analyses); use of whereas (while)

Referee #3 (Remarks to the Author):

Zbinden et al provide evidence that Shh upregulated expression of Nanog contributes to the growth of gliomas. The authors document expression of Nanog in Gliomas and perform experiments suggesting that Nanog is necessary for the propagation of glioma cells and their tumorigenicity in vivo. The authors also provide evidence that Shh-Gli activity regulates Nanog and that this is modulated by p53. These are novel data that will be of interest to the community, however there are some limitations to the data.

A difficulty with the data is inability of the authors to identifying Nanog protein expression in individual cells in a population of Glioma cells. This leaves open the question of whether Nanog is required cell-autonomously in tumor initiating/stem cells or is required non-autonomously in cells that support tumor growth. In attempt to circumnavigate this problem, the authors take advantage of a promoter fragment from the Nanog gene that appears to direct expression in a Nanog-like manner. This is used to support the contention that Nanog is expression in a small fraction of cells in a gliomasphere (Fig 1G), but the activity of the transgene is not compared directly to the Nanog antibody (Fig 1C). In addition using this reporter to FACS isolate cells expressing the reporter and comparison of these cells to those that lacked reporter activity did not reveal marked differences in behvior(p7). Thus the problem of whether Nanog is required within the tumor forming cells remains.

This issue also raises questions about the significance of Nanog/P8 mRNA expression in tumor lines (Fig 1A). Only a fraction of the lines appear to have significantly increased levels of expression, but in the absence of data that addresses how mosaic this expression is, it is difficult to interpret the data.

The usefulness and interpretation of the 'red/green' competition assay is unclear (Fig 3). The authors show that cells in which Nanog are knocked down are unable to generate tumors. It would seem unsurprising, therefore, that these cells are outcompeted when coinjected with tumor cells containing Nanog. What does the competition assay add to this experiment? In addition, the authors state (p10) "Analyses of these mice confirmed the presence of tumors" - I assume this refers to the control mice injected with GFP+only cells, but its is not clear from the sentence.

The observation that there are potential Gli binding sites in the Nanog promoter (p11) is of limited value without any functional testing of these sites.

Surprisingly, Ptch1 expression appears to be unaltered by Nanog reduction (Fig 5A). Ptch1 is generally considered to be a direct readout of Hh signaling and Gli1 activity. The lack of an effect on Ptch1 expression would appear to contradict the conclusion that Nanog affects the level of Hh signaling. The use of a reporter of Gli activity (as the authors have in previous papers) in the experiments described for Nanog and p53 in Fig 5 would be informative. The authors model (Fig 8) involves feedback between Gli1, Nanog and p53. Given the importance that the authors place on Gli1 in the Shh response in gliomas, alterations in Gli1 would be expected to affect the Shh-Gli transcriptional response and this would be expected to alter Ptch1 levels. An explanation of why Ptch1 is unaffected in these experiments is necessary to support the model.

1st Revision - authors' response 17 May 2010

Major experimental changes in the paper:
1- New functional data on NANOGP8 in vitro and vivo is provided in the new Figure 5. The new data shows that NANOGP8 is the main source of NANOG and is required for GBM proliferation and tumorigenicity, resolving the contribution of this gene.
2- New immunocytochemical analyses with a second antibody are now provided for NANOG protein, shown in Figures 1, S1.
3- New Western blot analyses for NANOG proteins are now provided in Figures 2,5 and 7.

4- New analyses of CD133 sorted cells is provided in the new Figure 3, showing that CD133+ cells harbor enriched expression of NANOG, NANOGP8 and NANOG->RFP. Importantly, we now show that CD133+ cells preferentially require NANOG function.
5- New confirmation of the regulation of NANOG protein by HH-GLI signaling is provided in Figure 7.
6- New demonstration of the regulation of p53 protein levels by NANOG is provided in Figure 6.
7- New GLI-dependent luciferase assays are shown in Figure 6 demonstrating the requirement of NANOG for GLI activity.
8- New rescue analyses of double knock-down of NANOG and p53 are shown in Figure 6. GLI1 levels are rescued by simultaneous blockade of p53 and NANOG.
9- New assays with temozolomide, the gold standard of care for GBM patients, and NANOG kd are provided in Figure 6. The results show that NANOG kd, which we demonstrate boosts p53 levels, dampens the effect of simultaneous temozolomide treatments, which act in part by activating p53.

Major presentation changes in the paper:
1- The entire paper has been re-written, correcting grammar, typos and the structure in general.
2- Additional details have been added to the legends as requested.
3- As per your request, the paper has been streamlined with two large experimental results figures being moved to supplementary material: The in vivo assays with adherent GBM cells, now Figure S2, and the in vivo epistatic assays with NANOG and HH-GLI in GBM-12, now Figure S3.

Detailed replies to specific major and minor points of the referees:

Referee #1

1) Although the loss-of-function data are strong, the inability to differentiate between Nanog1 vs. NanogP8 does not allow determination of their relative contributions to gliomagenesis. It is also not apparent whether the two NANOG1 'alleles' are valid alleles.

The two NANOG alleles are described in the sequence databases as mentioned in our paper. We have not designated them as such on our own. These differences are based on conserved changes that we indeed detect in our sequence analyses. We now include a description of why they are called alleles and give their sequence references (see Booth and Holland, 2004).

The issue of the contribution of NANOG versus NANOGP8 is indeed important. We have now managed to differentiate NANOGP8 specifically by RT-qPCR, and provide data with a NANOGP8-specific shRNA that NANOGP8 is the major contributor to NANOG protein in GBMs. Moreover, we have performed a new study with a NANOGP8-specific shRNA and show that it is essential in GBMs in vivo. Together, these results resolve the contribution of NANOGP8.

2) NANOGP8 is expressed (apparently) several fold higher than NANOG1 at the RNA level and further, the NANOG1-RFP reporter is not sufficient to track all clonogenic GBM cells. Since NANOGP8 promoter also exhibits putative GLI1 cis-elements, authors should focus their main efforts on the contribution from this locus.

As mentioned above, we now present a new Figure 5 on the dissection on the role of NANOGP8 in GBMs. We find that it is absolutely critical for GBM growth in vivo and that has the same effects as NANOG/P8 in vitro on GBM cell proliferation. Moreover, we show that a NANOGP8-specific shRNA abolishes NANOG protein production. This, together with the abundance of NANOGP8 as compared with NANOG, argue for a major role of NANOGP8, which we now functionally address.

3) The usefulness of the NANOG-RFP reporter is questionable. Clearly, a variant of NANOG mRNA is present in both subsets of RFP+/FRP- cells and both exhibit similar clonogenic potential. Are NANOG-RFP cells enriched in CD133? Inverse correlation with differentiation markers? Without further information this data is dispensable. The RT-PCR results in Fig. 1F are difficult to interpret.

NANOG->RFP is exceedingly useful to track NANOG mRNA expression as the more abundant NANOGP8 masks its signature. Moreover, since we can detect NANOGP8 but not NANOG singly by RT-PCR, we use the NANOG->RFP reporter to track the latter. However, we agree with this

referee that additional data was required to further substantiate a role of NANOG in stem cells. For this, and as requested, we have performed a number of new experiments, shown in the new Figure 3. We show that NANOGP8 is enriched in CD133+ cells as compared with CD133- cells in primary GBM-8; that NANOG->RFP is also enriched in CD133+ cells; that CD133+ cells are more sensitive than CD133- cells to blockade of NANOG in short-term proliferation assays and, finally, that CD133+ cells are more affected than CD133- cells in red/green competition assays blocking NANOG function in vitro. These data thus support the original conclusion that NANOG function is present but not restricted to GBM stem cells, nicely corroborating the original clonogenic and proliferative results. We have deleted the old Fig. 1F as suggested, since one allele of NANOG is difficult to track by RT-PCR as the product co-migrates with the band of the more abundant NANOGP8.

4) Detection of endogenous NANOG protein in GBM is necessary to convince readers of the authors' findings. Along the same line, the inability to analyze NANOG protein leaves the question open whether shp53 and/or shPTCH1, which upregulate NANOG at the RNA level, also regulate NANOG protein.

Following this reviewerís suggestions we have worked hard to identify reliable NANOG antibodies. We have tested a total of 9 anti-NANOG antibodies. We report data on the original one, a polyclonal antibody kindly made available by the Yamanaka lab but that seems to be exhausted, and new data with a polyclonal antibody from Kamiya that reliably recognizes NANOG in cells and in Western blots. As we previously noted for GLI1 (Stecca and Ruiz i Altaba, 2009 EMBO J), many commercial antibodies to NANOG appear to be unreliable, at least in human tumor cells. We chose to work with the Kamiya Ab since it recognizes the exogenous and endogenous proteins and NANOG is lost with specific shRNAs. Immunolocalization is shown in the new Figures 1,S1 and Western blots are shown in Figures 2,5,7. In addition, and as suggested, we have confirmed that altering the levels of HH-GLI signaling leads to altered NANOG protein levels. This is shown in the Western blot in the new Figure 7. Interestingly, we note that the protein changes for NANOG and GLI1 appear to be more drastic than those of the mRNAs after shRNA knock-down, possibly pointing to both RNA stability and translation effects. These results strengthen our original findings and conclusions.

5) There is no direct evidence that Nanog, Gli, and p53 cross-regulate one another.

We agree with this referee. We do not provide evidence for direct regulation but we mention in the text that we do not attempt to do so. Perhaps this referee considers cross-regulation only at the DNA-binding-transcriptional regulation level. As stated in the text we focus on functional (genetic) interactions and epistasis. While we agree that we do not provide (nor aim to provide in this paper) the kind of ChIP data one could wish to see to claim direct regulation, mostly given the lack of reliable anti-human GLI1 antibodies in large enough quantities, we provide very detailed in vitro and in vivo data on the different interactions. We note, however, that A. Gulinoís paper (which we have not read) appears to have ChIP data that confirm direct binding of mouse Gli1 to Nanog in mouse cells. In the text we now make very clear that the functional interactions we describe may be direct or indirect. This, however, does not detract from the value of our work and the elucidation of a fascinating and important functional network, which we now further substantiate with rescue experiments with double knock-downs of p53 and NANOG and with Western blot analyses shown in Figures 6 and 7. The network we describe is likely to be the focus of many future analyses and publications and we cannot be expected to resolve all issues in one paper.

Minor points:

1) The reported locations of putative Gli-binding sites seem to be inaccurate.

We thank this referee for this precise point. We have revised our data and it appears to be correct as per published sequences in Ensembl : ENSG00000111704. However, since we do not test them functionally, we have deleted mention of their exact location.

2) qPCR quantification and the y-axis NANOG mRNA levels (relative to?) need be clarified.

We now clarify this by stating that it is normalized expression.

3) The manuscript has many typographical errors. Reference 'Varnat et al., 2009' was not cited. Legends generally lack sufficient experimental detail.

The paper has been re-written and special attention has been given to typos. Varnat et al. is now included and the details of the figure legends expanded.

Referee #2

The authors use glioblastoma cells/ cell lines, which are already published, but the reference (Clement 2007) contains only rudimentary molecular data of these glial tumours. The authors should provide molecular details of these gliomas, in particular in the light of their variable growth, their claim of all GBM having a p53 mutation etc. Since the publication of the Clement's study in 2007, the IDH-1 and IDH-2 genes were discovered as being causative in the pathogenesis of most low grade gliomas, and consequently are also mutated in secondary GBM, in contrast to primary GBM, which typically do not have this mutation. In particular the moderate discrepancies within the GBM group (GBM1 and 10 vs. GBM-8 and GBM10-17) and compared to the Astrocytomas / Oligodendroglioma group may be correlated to the IDH mutation status.

Following this refereeís suggestions, we have sequenced IDH1 and IDH2 in the tumors we use functionally in the paper. We have found a single case with an IDH1 mutation. We now report this in the text. However, given that we have found only one case, we do not feel comfortable on expanding the interesting points raised by this referee in the paper.

The authors should also document the p53 status (mutation, LOH17p) of all their tumours and the 1p19q status at least of the oligodendroglial tumours.

As requested, we have also sequenced p53, focusing on the region containing the majority of hotspots, and report the mutations found in the new tumors. Since there is precious little oligodendroglial tumor material left we were unable to perform the chromosomal status analysis suggested. These analyses, however, do not change the functional results in our study. The mutational analysis this referee points to will indeed be critical in future studies when very large cohorts of primary tumors are tested for behavior following NANOG kd.

Minor points:

- *P7: did the authors examine this "equilibration" of Nanog positive and negative cells? Our experience with CD133+ and CD133- MACS sorted cells showed that enriched or depleted populations return to equilibrium when passaged in vitro. Did the authors observe a similar effect (with CD133+, or with NANOG+ cells)?*

This is a very interesting point that deserves full attention that, however, cannot be given in the present study. We have noticed that there is a tendency for the RFP+ and RFP- populations to equilibrate, as mentioned in the text, but have not performed more exhaustive studies since such analyses should optimally be performed with both NANOG and NANOGP8 reporters and the latter is not yet available. Moreover, we have not tested for equilibrium after long-term culture since we try to keep such cultures to the shortest times possible. Future studies should fully address this very interesting point.

- *Figure 1D: The legend refers to GBM-8, but the figure shows GBM12 in addition. Please clarify.*

All legends have been rechecked, corrected and expanded.

- *Figure 2B, D, F: How many spheres were counted to generate these data?*

We now explain in the text that >900 clonogenic events were tested per condition.

- *P5: "Analysis of NANOG protein were [sic!] hampered by the low levels of expression"; do the authors in fact mean that the cells express Nanog at low levels or instead that Nanog is*

expressed in a small number of cells? Their IF in Fig 1C suggests reasonably good expression in a small number of cells. Clarify, please.

We have now expanded our analysis of NANOG protein and have tested a large panel of antibodies. We find that NANOG is nearly ubiquitously expressed in GBM cells but that a small number express it at higher levels. We also provide Western blot analyses of NANOG protein.

- *P5 and Fig 1D: what is the profile of negatively sorted (CD133-) cells? Do they show a reduced Nanog expression? Di the authors carry out a FACS analysis with CD133 and their Nanog reporter? If they did, it may be interesting to show these data, to see the proportion of the following fractions: [CD133+, Nanog+]; [CD133-, Nanog-], [CD133+, Nanog-] and [CD133-, Nanog+] .*

The old Figure 1D (now Figure 3A) reported expression ratios, that is, mRNA expression values in CD133+ over CD133- populations after normalization with two housekeeping genes. We have now performed CD133 sorting and analyzed expression of NANOG/P8 and NANOGP8, and show in the new Figure 3 that there is enrichment in CD133+ cells. Similarly, in Figure 3 we also show that NANOG->RFP is enriched in CD133+ cells as compared with the CD133- population.

A) Nomenclature

- *: As a matter principle, the grade of all tumours should include the grading scheme, in this case probably the WHO grading scheme (e.g. Astrocytomas, WHO Grade III) .*

We have corrected the nomenclature in the text as suggested.

- *The term Astrocytoma does not exist according to the WHO classification and should not be used.*

For accessibility to non-experts, and given the extended use of astrocytoma in the literature, we have decide to use both nomenclatures but keeping them separately. In brakes we now provide the WHO classification.

- *The expression "lower grade gliomas (astrocytomas and oligodendrogliomas grade II and III)" is misleading. Although used here comparatively to GBM, it also wrongly suggests that WHO Grade III is a low grade, which is factually incorrect. WHO Grade III Astrocytomas and oligodendrogliomas are high grade by definition. The results section should mention the number of tumours assessed. The medulloblastoma can be omitted, as only one sample was used.*

We have corrected the problematic sentence to make sure that grade III and II are mentioned as lower grade gliomas in comparison to GBMs. We agree that a grade III is a high grade per definition. However, we have kept the medulloblastoma as a control.

- *The first use of the term "GBM-8 cells" without introducing the nomenclature is confusing to the reader unfamiliar with the experimental setup. The author should ensure the term GBM -8 is consistently used (not GMB-8) .*

We now mention what the Arabic numerals refers to in the text. We trust this will be acceptable.

- *Fig. 1A check AGII-1 (should read AII-1)*

This is now corrected.

B) Syntax, Grammar:

We thank this referee for the careful reading of the manuscript. We have now re-written all sections. We trust that we have amended all the syntax and grammar faults s/he found. In addition, we are hopeful that the excellent native copy editors will correct any linguistic problems non-native speakers make before publication.

- *General: The results section is poorly written. Non-exhaustive examples are given below:*
- *P6, 1st para: As expected,...*

- *P6, para2: This result ... (this sentence is incomprehensible); larger gliomaspheres, ... larger in adherent cultures ...*
- *P7: Special location (?) specific?*
- *P8: para3: Difficult syntax*
- *P9: What do the authors mean with "challenged"?*
- *P9: not clear what "same tumours in the same mice" means.*
- *P10 "NANOG is essential for GBM's": essential for propagation of GBM tumour cells..*
- *P10: consistent(ly) diminished expression (adverb)*
- *General: Analysis (not analyses); use of whereas (while)*

- *P15: "endow GBM stem cells with their multiforme character: all astrocytic can have a variable phenotype, not only the GBM. The GBM is no more "multiforme" than different types of WHO grade II astrocytomas!*

We have now corrected this issue.

Referee #3

A difficulty with the data is inability of the authors to identifying Nanog protein expression in individual cells in a population of Glioma cells. This leaves open the question of whether Nanog is required cell-autonomously in tumor initiating/stem cells or is required non-autonomously in cells that support tumor growth.

We now report analyses of NANOG protein after a major search of reliable antibodies. The data with replication incompetent lentivectors and the results of the red/green assays indicate cell-autonomous requirement. Moreover, we now clearly document different levels of expression but an apparently ubiquitous expression at lower levels. This argues for a requirement in all cells although we now show that CD133+ cells are more sensitive, consistent with the clonogenic assays.

In attempt to circumnavigate this problem, the authors take advantage of a promoter fragment from the Nanog gene that appears to direct expression in a Nanog-like manner. This is used to support the contention that Nanog is expression in a small fraction of cells in a gliomasphere (Fig 1G), but the activity of the transgene is not compared directly to the Nanog antibody (Fig 1C).

Following the results with the new Kamiya anti-NANOG antibody (KAb), we have performed additional experiments with NANOG->RFP and now show that the NANOG->RFP reporter is expressed at high levels in only a few cells, but at low levels in general manner, consistent with the antibody data. However, it is not possible to perform a direct correlation with total NANOG protein as NANOG antibodies will reveal NANOG made from both NANOG and NANOGP8, whereas the reporter tracks only expression from the NANOG locus.

In addition using this reporter to FACS isolate cells expressing the reporter and comparison of these cells to those that lacked reporter activity did not reveal marked differences in behvior(p7). Thus the problem of whether Nanog is required within the tumor forming cells remains.

The reason why RFPhigh- cells form spheres likely due to the fact that the NANOG->RFP construct does not track the expression of the more abundant transcripts from NANOGP8, which also make NANOG. We now provide several lines of evidence to suggest that NANOG function is required in GBM cells for proliferation and GBM stem cells for clonogenicity, and critically for all tumor growth in vivo. We also show that NANOG->RFP and NANOGP8 are enriched in CD133+ cells, and that these are more sensitive to NANOG kd than CD133- cells. Together, the data argue for a requirement of NANOG in GBM stem cells BUT also in cells of the tumor bulk, paralleling the requirement of GLI1 we documented previously (Clement et al., 2007; Stecca and Ruiz i Altaba, 2009).

This issue also raises questions about the significance of Nanog/P8 mRNA expression in tumor lines (Fig 1A). Only a fraction of the lines appear to have significantly increased levels of expression, but in the absence of data that addresses how mosaic this expression is, it is difficult to interpret the data.

The new finding of NANOG expression in all GBM cells should now resolve this issue. Moreover, we now report that NANOGP8 is the major contributor to NANOG protein based on the depletion of the latter in cells expressing a specific shRNA to NANOGP8. Moreover, we now provide new data that show that NANOGP8 is critically required for GBM growth in vivo. What remains to be addressed in future studies out of the scope of a single paper is the nature, stability and role of high expressors.

The usefulness and interpretation of the 'red/green' competition assay is unclear (Fig 3). The authors show that cells in which Nanog are knocked down are unable to generate tumors. It would seem unsurprising, therefore, that these cells are outcompeted when coinjected with tumor cells containing Nanog. What does the competition assay add to this experiment?

The two assays are different and complementary and we see great value in obtaining consistent data with alternative approaches. For example, in the case when we inject 100% GFP+ GBM cells expressing shNANOG and no tumors develop, the cells are not in a ëtumorí environment. Indeed, it could have been the case that a tumor environment made by the unaffected RFP+ control cells in the red/green assay would rescue non-cell autonomously the GFP+ shNANOG expressors. This is not the case, arguing for a strict requirement of NANOG independent of the environment. Conversely, it could have been that compromised GFP+ GBM cells expressing shNANOG would non-autonomously kill control RFP+ cells, thus causing a non-autonomous inhibition of tumor growth. We show this is not the case. Non cell-autonomous effects of NANOG has been recently reported (Nanog is required for primitive endoderm formation through a non-cell autonomous mechanism. Messerschmidt DM, Kemler R. Dev Biol. 2010). These issues are important and add to the detail and value of our work. Thus, we prefer to keep both assays in the paper.

In addition, the authors state (p10) "Analyses of these mice confirmed the presence of tumors" - I assume this refers to the control mice injected with GFP+only cells, but its is not clear from the sentence.

This is correct. It has now been clarified in the text.

The observation that there are potential Gli binding sites in the Nanog promoter (p11) is of limited value without any functional testing of these sites.

We are in full agreement and have made sure that this is reflected in the text. We simply point to their presence but for technical reasons (not enough specific Abs available against human GLI1) we have not embarked on testing direct binding. Whether the regulation is direct or indirect does not diminish in any way the value of our important functional assays in vitro and in vivo, or of the epistatic analyses we report. Moreover, this paper is co-submitted with an independent paper of the lab of A. Gulino where they appear to provide direct binding of mouse Gli to Nanog regulatory regions.

Surprisingly, Ptch1 expression appears to be unaltered by Nanog reduction (Fig 5A). Ptch1 is generally considered to be a direct readout of Hh signaling and Gli1 activity. The lack of an effect on Ptch1 expression would appear to contradict the conclusion that Nanog affects the level of Hh signaling.

This referee is correct in considering Ptch1 a direct Gli target. The work of the Toftgard lab and others have shown this to be the case in cultured cells. However, different targets have different kinetics and this is precisely what we show. The original assays were done 4 days post-transduction. We have repeated them analyzing Ptch1 expression at 3 days post-transduction and we find that Ptch1 is indeed downregulated at 3 days (Figure 6A). In addition, we provide Western blot data showing that GLI isoforms are undetectable after NANOG kd (Figure 6B). We can thus conclude that NANOG does indeed regulate HH-GLI signaling.

The use of a reporter of Gli activity (as the authors have in previous papers) in the experiments described for Nanog and p53 in Fig 5 would be informative.

As requested by this referee we have performed GLI-reporter luciferase assays and show that

NANOG is indeed required for GLI activity (Figure 6C). These results provide additional support for the conclusion that NANOG regulates HH-GLI activity.

The authors model (Fig 8) involves feedback between Gli1, Nanog and p53. Given the importance that the authors place on Gli1 in the Shh response in gliomas, alterations in Gli1 would be expected to affect the Shh-Gli transcriptional response and this would be expected to alter Ptch1 levels. An explanation of why Ptch1 is unaffected in these experiments is necessary to support the model.

As mentioned above, we provide now clear data on the regulation of Ptch1 by NANOG (see above). This should resolve this issue.

Additional Correspondence 01 June 2010

Thank you for sending your revised manuscript. Two of the original referees have assessed it again. You will be pleased to learn that in their view the criticisms have been addressed satisfactorily. You will soon receive a formal acceptance letter.

Before that, please send a modified abstract that we can incorporate in ot the final version as indicated by ref#3.

Thank you very much again for considering our journal for publication of your work.

With best regards

REFEREE REPORTS

Ref#2

1) The authors have addressed most of my comments. I am happy that the article can be published as is.
2) The publication of both articles back to back make sufficient impact to justify an Editorial or highlighting them. This topic is of significant general interest.

Ref#3

The authors have addressed each of the points raised in my initial review. This has included substantial amounts of new data.

The new data satisfactory address the areas of concern.

I remain surprised that Ptch1 does not behave as might be expect but accept that the kinetics of the response might explain this discrepancy.

Connecting Shh signaling, Nanog and p53 in glioblastoma will be of significant interest to the field. The data make a convincing case that Nanog is essential for the Shh-Gli dependent response of gliomas.

Minor point:
The last line of the Abstract needs attention.

