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CAMTA1 is a novel tumor suppressor regulated by miR-9/9* in glioblastoma stem cells

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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 01 July 2011

Thank you very much for submitting your research paper that identifies CAMTA1 as novel tumor suppressor regulated by miRNA's in glioblastoma cells for consideration to The EMBO Journal editorial office.

Having received consistent and very positive comments from two expert scientists, I am able to reach a decision on your study to facilitate efficient proceedings. Despite the expressed enthusiasm, I would still be grateful if you consider their thoughtful remarks and adopt/integrate necessary amendments into a revised version of your study.

Please be reminded that it is EMBO_J policy to allow a single round of revisions only and that the final decision does depend on the content of the final version of your manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

Schraivogel and colleagues identified CAMTA1 as a target of miR-9/9* in glioblastoma and provide solid evidence that CAMTA1 has tumour suppressor functions in these cells. The authors nicely demonstrate that miR-9/9* is strongly induced in a glioblastoma cancer stem cell population where it regulates neurosphere formation through repression of CAMTA1. Through a series of in vitro and

in vivo experiments, the tumour suppressive function of CAMTA1 is demonstrated. The manuscript is well written and the results are clearly presented. I only have minor comments.

minor comments:

1. typo p4 (Introduction): ... can give rise to functional miRNAs.
2. Throughout the manuscript, p-values are missing for most of the figures. These should be included (either in the figure or in the text).
3. Figure 1F, panel3: Not clear why authors use miR-34a as a control. Based on Figure 1D, one would expect that miR-103, miR-16 or miR-27b are the most obvious controls.
4. Figure 1F, panel 1 and 2: I would suggest performing a group comparison (CD133+ vs CD133- cells) in order to have a p-value demonstrating that miR-9/9* is significantly higher expressed in CD133+ cells derived from these different cell lines compared to CD133- cells.
5. The authors argue that some cell lines show mild differences in miR-9/9* expression and that this might be due to different tumour origin. Could it be that the small RNA that is used for normalisation of the qPCR expression data is not stable (and higher expressed in these cell lines)? This issue could be addressed by including multiple reference genes and evaluating their stability.
6. The type of controls that were used in Figure 2 should be specified in the manuscript (scrambled, commercial, ...).
7. On page 8, the authors state that miR-9 inhibits differentiation however this is based on the expression of a single marker. The authors should either evaluate additional markers to support this claim, evaluate differentiation morphologically or tone down their statement.
8. Figure 3B: Why not rank the genes according to the fold change in enrichment between miR-9 and miR-122. In that way, the most promising candidates would be ranked on top. On a side note, What if the difference in fold enrichment between miR-9 and miR-122 is not due to a depletion in the miR-9 sample but due to an increase in the miR-122 sample? After all, miR-122 is not a true negative control.
9. Figure 4A: no obvious difference between colours for conserved and non-conserved miR-9 seeds
10. On page 9 the authors state that functional evidence for a role for CAMTA1 as a tumour suppressor is still missing however in the discussion they refer to a paper by Heinrich describing tumour suppressor functions for CAMTA1.
11. Figure 7C: no green curve is present in the plot. How do the authors define 'upregulated' in this analysis? This is not entirely clear from the text.
12. Page 10: The authors should use the official gene symbol for ANF, as they do in Figure 8.
13. In the discussion on page 13, the authors refer to the paper of Heinrich stating that CAMTA1 is a tumour suppressor in neuroblastoma. The finding that CAMTA1 is a target of miR-9 is interesting in this respect as miR-9 has been demonstrated to be a target of MYCN (Ma et al, Nature Cell Biology, Mestdagh et al., Oncogene). MYCN amplification is closely associated with 1p36 deletions in neuroblastoma and could therefore, through the induction of miR-9, further repress CAMTA1 expression.

Referee #2:

This manuscript reports the results of an investigation into the miRNA expression profile of CD133+ glioma stem cells (GSCs). Sequencing-based profiling identifies miR-9/9*, miR-17, and miR-106b as miRNAs whose expression levels are highest in GSCs relative to CD133- cells. The authors then embark on a detailed functional characterization of these miRNAs in the context of glioma stem cell biology and glioma pathogenesis. Their most significant finding involves the identification of CAMTA1 as a target of miR-9/9* and miR-17. miR-9/9* and miR-17 knockdown are each found to inhibit neurosphere formation in GSCs, a phenomenon that is recapitulated by CAMTA1 expression. Furthermore, CAMTA1 expression hampers tumor formation when these same GSCs are xenografted into mice. Finally, the authors mine existing data from REMBRANDT and TCGA to show that decreased CAMTA1 levels are associated with higher grade astrocytomas and unfavorable prognosis in primary glioblastoma, the latter a correlation that they also make with ANF, a primary downstream effector of CAMTA1.

This manuscript is well-written and the data are presented fairly without overinterpretation. The findings are significant in that solid connections are established between miRNA biology, the maintenance of stem-like character, and glioma pathogenesis via a defined regulatory interaction. As such, this work would be of general interest to the readership of the EMBO Journal.

I have some minor suggestions that I believe would significantly strengthen the paper.

1. The identification of CAMTA1 repression as the mechanism of action of miR-9/9* and miR-17 is particularly striking given what is known about oligodendroglioma and the frequent 1p/19q chromosomal codeletion that has been associated with low- and high-grade variants. This should be discussed, in particular, the recent localization of the minimal deleted region on chromosome 1p to the CAMTA1 gene (1). Furthermore, miR-9 expression has already been correlated with WHO grade in oligodendroglioma (2).

2. In a similar vein, the authors should perform REMBRANDT-based survival analysis comparing low and high CAMTA1 expressing oligodendrogliomas. Intriguingly, given the association of 1p/19q codeletion with better prognosis in oligodendroglioma, the authors may find that CAMTA1-low oligos actually outperform their CAMTA-high counterparts, in sharp contrast to their results for GBM. This would not, however, decrease the significance of their findings in my mind.

3. Of more minor concern, the authors should make sure to explicitly state exactly which cell lines are used in which experiments.

4. In the introduction, the sentence "Since miRNAs are fundamental regulators of basic cellular processes such as cell cycle control, cell differentiation and proliferation, miRNAs are frequently de-regulated in tumors." should be re-written as "MiRNAs are fundamental regulators of basic cellular processes such as cell cycle control, cell differentiation and proliferation, and are frequently de-regulated in tumors."

References

1. Barbashina V, Salazar P, Holland EC, Rosenblum MK, Ladanyi M (2005) Allelic losses at 1p36 and 19q13 in gliomas: correlation with histologic classification, definition of a 150-kb minimal deleted region on 1p36, and evaluation of CAMTA1 as a candidate tumor suppressor gene. Clin Cancer Res 11:1119-1128
2. Nelson PT, Baldwin DA, Kloosterman WP, Kauppinen S, Plasterk RH, Mourelatos Z (2006) RAKE and LNA-ISH reveal microRNA expression and localization in archival human brain. RNA 12:187-191

1st Revision - Authors' Response 13 July 2011

We respond to the reviewers' comments as follows:

Reviewer#1:
1. typo p4 (Introduction): ... can give rise to functional miRNAs.
We have changed the typo accordingly.

2. Throughout the manuscript, p-values are missing for most of the figures. These should be included (either in the figure or in the text).
We have added p-values to all the Figures as requested by reviewer#1.

3. Figure 1F, panel3: Not clear why authors use miR-34a as a control. Based on Figure 1D, one would expect that miR-103, miR-16 or miR-27b are the most obvious controls.
We have used miR-34a because this miRNA has also been implicated in cancer and it is stronger expressed in CD133- cells in our deep sequencing data. miR-34a shows differential expression in

the different cell lines and it is indeed stronger expressed in negative cells in some cell lines. However, the expression levels appear to be more random. Our data suggest that the cell lines might derive from different origins and are probably not fully comparable with regard to their miRNA profile. We have mentioned this in the text and we would expect similar results by using other controls from the miRNA list.

4. Figure 1F, panel 1 and 2: I would suggest performing a group comparison (CD133+ vs CD133- cells) in order to have a p-value demonstrating that miR-9/9 is significantly higher expressed in CD133+ cells derived from these different cell lines compared to CD133- cells.*
As suggested, we have performed group comparisons using one-way ANOVA. We find that miR-9 is significantly higher in CD133+ compared to CD133- cells (p=0.0393). For miR-9* we calculated p=0.0793, which would not be statistically significant. As expected for miR-34a, which is differentially expressed in the different cell lines, p=0.3392.
Although the performed group comparison provides useful information, we would like to show the individual cell lines in Figure 1F. In cell line R54, for example, the miR-9/9* differences between CD133+ and CD133- cells are rather marginal. This, however, is important information, which would not be visible in a group comparison.

5. The authors argue that some cell lines show mild differences in miR-9/9 expression and that this might be due to different tumour origin. Could it be that the small RNA that is used for normalisation of the qPCR expression data is not stable (and higher expressed in these cell lines)? This issue could be addressed by including multiple reference genes and evaluating their stability.*
We agree that we cannot exclude that the small RNA (U6) has different stabilities in the different cell lines. However, we have performed Northern blotting against a tRNA (see also Figure 1E) and we did not observe any differences in RNA stability between CD133+ and – cells. Furthermore, the absolute Ct values of U6 would also argue against specific destabilization of the U6 RNA. Therefore, we believe that the observed effects are not due to technical differences and the most plausible explanation would be that the cell lines might have different origins as we discus in the manuscript.

6. The type of controls that were used in Figure 2 should be specified in the manuscript (scrambled, commercial, ...).
We have now added the specific controls that have been used to the respective figure legends.

7. On page 8, the authors state that miR-9 inhibits differentiation however this is based on the expression of a single marker. The authors should either evaluate additional markers to support this claim, evaluate differentiation morphologically or tone down their statement.
We agree with reviewer#1 that one single marker is probably not enough for our statement. However, Tuj1 is widely used as marker for neuronal differentiation. On page 8 of our manuscript, we state "…our data suggest…", which we believe is not too exaggerated. Nevertheless, we have revised the statement as follows: "In summary, our data suggest that miR-9/9* help maintaining CD133+ cells probably by preventing differentiation."

8. Figure 3B: Why not rank the genes according to the fold change in enrichment between miR-9 and miR-122. In that way, the most promising candidates would be ranked on top. On a side note, What if the difference in fold enrichment between miR-9 and miR-122 is not due to a depletion in the miR-9 sample but due to an increase in the miR-122 sample? After all, miR-122 is not a true negative control.
In the revised version of Figure 3, we have ranked the identified targets accordingly.
miR-122 is a liver-specific miRNA and it is not expressed in our cell lines. Therefore, we hypothesized that a 2'-O-methylated inhibitor against miR-122 might be a useful negative control. Although we cannot fully exclude that the observed fold changes are due to an increase in our control, we can clearly validate CAMTA1 as miR-9* target. In addition, we have also used miR-301 as control for the validation of CAMTA1 as miRNA target. With the CAMTA1 characterization we therefore validate the specificity of our target identification approach.

9. Figure 4A: no obvious difference between colours for conserved and non-conserved miR-9 seeds
We have changed the colors accordingly.

10. On page 9 the authors state that functional evidence for a role for CAMTA1 as a tumour suppressor is still missing however in the discussion they refer to a paper by Heinrich describing tumour suppressor functions for CAMTA1.
We have changed the text on page 9 to "It has been suggested that CAMTA1 functions as tumor suppressor in neuroblastoma (Henrich et al.; Finkler et al.). However, a link between CAMTA1 function and glioblastoma has not been reported so far."

11. Figure 7C: no green curve is present in the plot. How do the authors define 'upregulated' in this analysis? This is not entirely clear from the text.
We have changed green to blue. Thank you very much for pointing this out. The definition of *upregulated* is included in the Figure.

12. Page 10: The authors should use the official gene symbol for ANF, as they do in Figure 8.
We have changed ANF to NPPA as suggested by reviewer#1.

13. In the discussion on page 13, the authors refer to the paper of Heinrich stating that CAMTA1 is a tumour suppressor in neuroblastoma. The finding that CAMTA1 is a target of miR-9 is interesting in this respect as miR-9 has been demonstrated to be a target of MYCN (Ma et al, Nature Cell Biology, Mestdagh et al., Oncogene). MYCN amplification is closely associated with 1p36 deletions in neuroblastoma and could therefore, through the induction of miR-9, further repress CAMTA1 expression.
We thank reviewer#1 for this valuable information. We have added the following statement to our discussion: "Interestingly, miR-9 expression is stimulated by MYCN in breast cancer and MYCN is closely related to the frequent 1p36 deletion {Ma, 2010 #3103}{Mestdagh, 2010 #3939}. It is tempting to speculate that MYCN might contribute to miR-9/9* expression in glioblastoma cells as well."

Referee #2
1. The identification of CAMTA1 repression as the mechanism of action of miR-9/9 and miR-17 is particularly striking given what is known about oligodendroglioma and the frequent 1p/19q chromosomal codeletion that has been associated with low- and high-grade variants. This should be discussed, in particular, the recent localization of the minimal deleted region on chromosome 1p to the CAMTA1 gene (1). Furthermore, miR-9 expression has already been correlated with WHO grade in oligodendroglioma (2).*
We thank reviewer#2 for the information, which we have added to our manuscript. We state now in the discussion: "Very recently, it has been demonstrated that among others, expression levels of miR-9 and miR-17 are correlated with malignant progression of gliomas (Malzkorn et al, 2010). In addition, miR-9 has been implicated in oligodendroglioma {Nelson, 2006 #3940}. This supports our finding that these miRNAs are important for glioma pathogenesis."
We have also added: "Strikingly, a minimal deletion comprising only the CAMTA1 gene has been identified suggesting that CAMTA1 is indeed important for glioma formation {Barbashina, 2005 #3941}."

2. In a similar vein, the authors should perform REMBRANDT-based survival analysis comparing low and high CAMTA1 expressing oligodendrogliomas. Intriguingly, given the association of 1p/19q codeletion with better prognosis in oligodendroglioma, the authors may find that CAMTA1-low oligos actually outperform their CAMTA-high counterparts, in sharp contrast to their results for GBM. This would not, however, decrease the significance of their findings in my mind.
We have analyzed CAMTA1 expression in oligodendroglioma using the REMBRANDT database as reviewer#2 suggested. Indeed, CAMTA1 expression seems to correlate with reduced patient survival in oligodendroglioma (although the data obtained from REMBRANDT are statistically not significant). One plausible scenario could be that CAMTA1 does not function as such a clear tumor suppressor compared to glioblastoma. CAMTA1 is a putative transcription factor and it is well possible that it targets different genes and therefore it could have distinct functions in different cell types.

3. Of more minor concern, the authors should make sure to explicitly state exactly which cell lines are used in which experiments.
We apologize for not clearly pointing out which cell lines have been used. We have now added the cell lines to each figure legend.

4. In the introduction, the sentence "Since miRNAs are fundamental regulators of basic cellular processes such as cell cycle control, cell differentiation and proliferation, miRNAs are frequently de-regulated in tumors." should be re-written as "MiRNAs are fundamental regulators of basic cellular processes such as cell cycle control, cell differentiation and proliferation, and are frequently de-regulated in tumors."
We have changed the text accordingly.

