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EGFL7 enhances surface expression of integrin α5β1 to promote angiogenesis in malignant brain tumors

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Transaction Report:

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

1st Editorial Decision   02 October 2017

Thank you for the submission of your manuscript "EGFL7 enhances surface expression of integrin α5β1 to promote angiogenesis in malignant brain tumors". We have now heard back from the two referees whom we asked to evaluate your manuscript.

As you will see, while the referees acknowledge the potential interest of the study and reviewer #1 is rather on the optimistic side, referee #2 is much more reserved regarding the clinical and translational potentials of the strategy reported here. In fact this referee also remained on his/her position during our cross-commenting exercise (please see below), which, I am afraid, preclude publication of the manuscript in EMBO Molecular Medicine.

"I agree with the statement of Referee 1 "Inhibition of EGFL7 diminished tumor growth improving the survival of mice that were orthotopically harboring gliomas". However, I remain doubtful regarding the likely medical impact of the reported results based on the following considerations:

1) VEGF blockade is the current best option for anti-angiogenic treatment of glioma. Yet, it can only slightly prolong survival and cannot achieve a cure: therefore therapeutic targets that improve anti-VEGF potential are sorely needed.

2) In the data reported, anti-EGFL7 treatment performs similarly to anti-VEGF, but crucially the combination is not better than any of the two alone: the survival curves for the combined treatment are not statistically significantly better than single treatments in both Fig. 6J and Suppl. Fig. 5J. Further, all vascular parameters analyzed in both figures are affected essentially in the same way by both individual treatments and by the combination, in full agreement with the lack of advantage over anti-VEGF alone.

3) On the other hand, it is possible to observe a significant survival advantage over anti-VEGF alone in the same model with other treatments (e.g. data by the group of Rakesh Jain with an antibody targeting both VEGF and Ang2: see Fig. 1 in Kloepper J. et al. PNAS 2016).

All in all, the data seem to suggest that EGFL7 and VEGF functions are not synergistic in glioma angiogenesis and so their individual targeting yields equivalent results, but does not show potential for improving the results of the gold-standard treatment with anti-VEGF alone even when combined.

Based on this, my evaluation is that the manuscript would be more suited for a journal with a more fundamentally oriented focus and less for EMBO Molecular Medicine, where the translational medical implications are center-stage."

I am sorry that the outcome for this manuscript could not have been more positive. I do want to emphasize, however, that this is not intended to imply a lack of interest on our part in either your work in particular or this field in general, and we hope that you will continue to consider EMBO Molecular Medicine for other submissions in the future when it seems appropriate.

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

Currently anti-angiogenic approaches for the treatment of gliomas have confronted with major hurdles. The finding by the authors that EGFL7 might perform as a druggable pro-angiogenic factor is important and could have clinical application.

Referee #1 (Remarks for Author):

The authors show that EGFL7 by modulating the expression of avb3 and a5b1 on the tumor endothelial cells promotes glioma angiogenesis driving growth and invasion. Inhibition of EGFL7 diminished tumor growth improving the survival of mice that were orthotopically-harboring gliomas.

Comments:
The major strength of this paper is the use of orthotopic glioma models to assess the function of EGFL7. In addition, they have provided mechanistic data attempting to uncover the mechanism by which targeting various integrins might collaborate to block glioma cell growth.

There are a few minor issues that need to be addressed before publication of this paper.

1) The combination of anti-VEGF-A along with targeting EGFL7 might significantly increase the toxicity of this regimen. Could the authors discuss and propose an approach that will diminish the toxicity associated with this combination therapy?

2) EGFL7 could interfere with Notch signaling through blocking the activities of various Notch ligands, such as Jagged1 and Dll4. Then targeting EGFL7 might unleash Notch signaling, such as Dll4 activation of Notch1 and Notch 4 that could lead to non-productive angiogenesis. Thus, does targeting EGFL7 affect Notch dependent endothelial sprouting?

3) The precise mechanism by which EGFL7-mediated upregulation of avb3 and a5b1 regulate glioma tumor growth should be better defined.

Referee #2 (Remarks for Author):

The Authors explored the role of the extracellular matrix protein EGFL7 in driving angiogenesis in malignant glioma, a type of tumor that is highly dependent on vascular invasion and is typically lethal, and its therapeutic potential to improve the unsatisfactory results of anti-angiogenic therapy

with VEGF blockade. They found that EGFL7 is expressed in the blood vessels of human glioma samples, but not by the glioma cells themselves. Implantation of glioma cell lines in mice lacking EGFL7 prolonged survival of mice, whereas the absence of the EGFL7-encoded parasitic miRNA 126/126* did not have an effect. Conversely, EGFL7 overexpression in the glioma lines shortened survival, causing increased vessel density and also vessel maturation. Further, EGFL7 stimulated in vitro endothelial sprouting by increasing surface expression of integrin α5β1 and reducing its endocytosis, possibly through interaction with integrin αvβ3. Lastly, treatment with an EGFL7 blocking antibody reduced both vessel density and maturation in experimental gliomas and prolonged the median survival time of implanted animals. This effect was similar to that of a VEGF blocking antibody and the combination, although modestly increasing median survival time, did not show significant synergistic effects.

This study addresses a significant problem, namely the lack of effective treatments for malignant glioblastoma, and reports some interesting results. However, a few considerations limit the general importance of the findings:

1) The medical impact appears modest. In fact, while the data show that EGFL7 does have a role in glioma angiogenesis, its targeting is not more effective than the established (albeit still unsatisfactory) approach of VEGF blockade and their combination does not significantly improve the outcome of anti-VEGF alone. Further, the combination treatment does not significantly improve any vascular parameter compared to anti-VEGF or anti-EGFL7 alone, including vessel density, suggesting there is no additive or synergistic anti-angiogenic effect, in agreement with the modest and not-significant effect on duration of survival.

2) The mechanistic part, showing the effects of EGFL7 on integrin recycling and in vitro endothelial sprouting, is limited in scope. On one hand, the data provide only an incremental step in knowledge compared to previous results by the same group, and on the other they fail to address the actual relevance of these findings for glioma therapy. Does targeting of α5β1 recapitulate the effects of anti-EGFL7 treatment in experimental glioma models? Or is the effect of EGFL7 targeting in gliomas due to other EGFL7 functions that have been described, for example its regulation of Notch signaling?

3) The proposed molecular mechanism that EGFL7 increases surface expression of α5β1 integrin through ligation with αvβ3 makes sense, but it would need to be proven by a loss-of-function experiment, showing that interference with αvβ3 by a blocking antibody or siRNA knockdown does prevent the effects of EGFL7 on α5β1.

4) The data show that EGFL7 targeting reduces vessel maturation and increases permeability, but no attempt is made to address how EGFL7 may regulate these processes.

Other points
1) Fig. 1B - It is unclear how to interpret this panel: why do red circles appear to indicate methylation in CpG island 1 and no methylation in CpG island 2?

2) Suppl. Fig. 1D - It would be useful to include a scale also in the lower part of the y axis to interpret the range of the black bars: do they reach to 1? 2? 5?

3) Suppl. Fig. 1E - I could not find any method for the derivation of organotypic spheroids from patient-derived GMB biopsies.

4) Suppl. Fig. 1F - The EGFL7 stains are difficult to interpret, possibly due to low magnification, and also appear to delineate dot-like structures rather than vessels, like evident instead in the histochemistries shown in Fig. 1E. Higher-magnification and clearer images would be helpful.

5) The conclusion that EGFL7 expression in glioma specimens was restricted to blood vessels is substantiated by the data, but that it occurred independently of miR-126/126* would be strengthened by similar analysis of its pattern of expression (or lack thereof) in the patient samples by in situ hybridization.

6) Suppl. Fig. 2B, C and F - The pattern of EGFL7 appears clearly suggestive of vascular structures, although the glioma cells are responsible for its overexpression in these over-expressing tumors. Curiously, instead, the patter in panel 2E is not vascular anymore. This localization should be verified by co-staining with endothelial and glioma markers and an explanation would be useful.

7) Fig. 5D-E - The provided MRI images appear to serve just a decorative function, since only one condition is shown and it is not actually mentioned which one it is. On the other hand, the graph in panel A is rather superfluous (intrastriatal injection and subsequent histological analyses are straightforward and were already described in a cartoon in Fig. 2A) and it could be removed, using the space to show complete MRI data for all conditions.

8) Figs. 2 and 5 analyze the same experiment and would best follow each other rather than being separated by the integrin data.

9) How were histological quantifications of vascular parameters actually performed? Vessel density is presented as cubic microns, which seems more a unit of volume than of density. What are the arbitrary units used to score vascular maturation? This information is missing from the methods and quite important to understand what is being measured and its significance. In fact, for example, just looking at the immunofluorescence images in Fig. 5 and Suppl. Fig. 4, pretty much all endothelial structures appear associated with collagen IV, SMA and PDGF-Rb signals in all conditions (except the single vessel shown in the control of Fig. 5G). This would suggest that there are no "naked vessels", but then quantifications in Arbitrary Units show dramatic differences for all these parameters.

Author's appeal   10 October 2017

Thank you for your consideration of our manuscript for EMBO Mol Med. I was pleased reading how much merit reviewer 1 found in our work. However, I was fairly surprised on the judgement of reviewer 2, which you eventually followed.

The reviewer's claim that any new anti-angiogenesis treatment of glioma can only be an additive to anti-VEGF treatment as the gold standard does not hold true for glioma patients. As we discuss in our manuscript the AVAglia and RTOG 0825 studies very convincingly show that anti-VEGF treatment remains palliative. The patients have no overall survival benefit but if at all their symptoms are slightly reduced within a narrow time window. Therefore bevacizumab (Avastin®), the best-known VEGF-inhibitor, has not been accredited for glioma treatment by the EMA in the EU. Only the FDA approved it in the US and it is used in Switzerland as a palliative treatment. In light of these studies it seems unlikely that anti-VEGF-treatment will remain a significant option for the treatment of malignant glioma in the EU. Certainly, it is far away from being a gold standard though it is still used in the absence of better alternatives, which are desperately needed as patients usually within one-year post diagnosis.

Anti-EGFL7-treatment seems quite promising in this context as it increased the survival time in our glioma models comparably to anti-VEGF treatment. Though reviewer 2 is right that the add on effect reached by a combination of both treatments (Fig. 6J and Suppl. Fig. 5J) was not highly significant (P=0.16 for the GL261 model and P=0.29 for U87), a strong tendency towards improvement of the anti-VEGF regimen by anti-EGFL7 could be observed. However, the potential improvement of anti-VEGF efficacy was just a side observation and we haven't followed up on it deeper as it was our goal to identify EGFL7 as a target for glioma treatment per se. If you would grant us the opportunity to increase the sample size of this particular experiment I am convinced that this difference would become statistically more significant as it is now.

In addition, the EGFL7 protein inherits some promising advantages over VEGF as a target molecule. EGFL7 is a secreted and barely soluble protein (comparable to fibronectin) which resides within the extracellular matrix of the tumor once the blood vessels are gone upon therapy. There it supports the regrowth of blood vessels along the trails of the former blood vessels during tumor recurrence. Blocking it delays blood vessel re-growth. In particular, this is relevant for malignant glioma as in this type of neoplasm the recurrence kills the patient, not the primary tumor. Therefore,

anti-EGFL7 treatment does not only act comparably to anti-VEGF treatment (and likely improves it) but in addition offers some remarkable advantages over it.

In light of these explanations I respectfully ask you to revisit your decision on our manuscript in order to give us the opportunity to present anti-EGFL7-treatment as a promising new option for the cure of malignant glioma. We believe that our findings are important for patients and will significantly contribute to their cure.

2nd Editorial Decision   30 october 2017

Thank you for your patience while we were reconsidering our decision. I am sorry it took so long, we are short in staff at the moment, hence the delay.

I have asked one of our external advisors about your paper and I am happy to disclose that this advisor agrees with you and referee 1 that the paper should be revised. Therefore, I'd like to invite you to revise the paper but please pay attention to referee 2's comments and address the commented limitations as much as possible. I would also like for you to try and address our advisor suggestions as we agree it would improve the clinical relevance of the findings.

"... the lack of a synergistic effect could be analyzed by studying the effect of EGFL blocking on VEGF production. Because anti-angiogenic approaches are not used as single agents in these tumors, it should be more interesting to compare the following arms: 1) standard chemo; 2) anti-VEGF; 3) anti- EGFL; 4) 2) anti-VEGF- chemo; 3) anti EGFL+chemo"

We would welcome the submission of a revised version within three months for further consideration and would like to encourage you to address all the criticisms raised as suggested to improve conclusiveness and clarity. Please note that EMBO Molecular Medicine strongly supports a single round of revision and that, as acceptance or rejection of the manuscript will depend on another round of review, your responses should be as complete as possible.

EMBO Molecular Medicine has a "scooping protection" policy, whereby similar findings that are published by others during review or revision are not a criterion for rejection. Should you decide to submit a revised version, I do ask that you get in touch after three months if you have not completed it, to update us on the status.

Please also contact us as soon as possible if similar work is published elsewhere. If other work is published we may not be able to extend the revision period beyond three months.

Please read below for important editorial formatting and consult our author's guidelines for proper formatting of your revised article for EMBO Molecular Medicine.

I look forward to receiving your revised manuscript.

2nd Revision - authors' response   26 May 2018

Editor (Remarks for Author):
Because anti-angiogenic approaches are not used as single agents in these tumors, it should be more interesting to compare the following arms: 1) standard chemo; 2) anti-VEGF chemo; 3) anti-VEGF anti EGFL chemo

Response to the editor
In order to address the interesting suggestion of the editor, we established a regimen using temozolomide as a chemotherapeutic agent in combination with anti-VEGF and anti-EGFL7 antibodies. Upon tumor engraftment, animals were treated twice a week with this combination therapy until the end of the experiment. Mice receiving anti-VEGF and anti-EGFL7 antibodies in combination with temozolomide survived significantly longer as compared to anti-VEGF (survival increased by about 7 d on average) or isotype control (survival increased by about 18 d on average)

treatment alone (new Fig. 6K). Data suggest a beneficial effect of EGFL7 treatment on standard glioma therapy and underpin the clinical relevance of our findings.

Referee #1 (Remarks for Author):
1) The combination of anti-VEGF-A along with targeting EGFL7 might significantly increase the toxicity of this regimen. Could the authors discuss and propose an approach that will diminish the toxicity associated with this combination therapy?

Response to the reviewer
The reviewer's point is well taken. Bevacizumab-specific toxicities such as hypertension or proteinuria manifest upon combination of the antibody with chemotherapy. The incidence of hypertension for example increases in a dose-dependent manner. Anti-EGFL7 treatment could serve to reduce the anti-VEGF concentration and thereby reduce the specific toxic side effects of bevacizumab. Furthermore, a combination of anti-VEGF/anti-EGFL7 might be applied to reduce the doses of chemotherapeutics. This point has been added to the discussion in the revised version of the manuscript.

2) EGFL7 could interfere with Notch signalling through blocking the activities of various Notch ligands, such as Jagged1 and Dll4. Then targeting EGFL7 might unleash Notch signalling, such as Dll4 activation of Notch1 and Notch 4 that could lead to non-productive angiogenesis. Thus, does targeting EGFL7 affect Notch dependent endothelial sprouting?

Response to the reviewer
The reviewer is completely right; EGFL7 affects angiogenesis in a Notch-dependent manner. This has been shown by Heidi Stuhlmann's group at Cornell University (Nichol et al., Blood, 2010). However, whether or not signalling mechanisms in physiological and pathological angiogenesis may be compared is still under debate. In particular, the role of Notch signalling in glioma per se has not been unravelled, yet. Others (Koch & Radke, Cell Mol Life Sci, 2007) and us (Teodorczyk & Schmidt, Front Oncol, 2015) elaborated on this topic in reviews as Notch may act as an oncogene but also as a tumour suppressor. In order to interpret our findings on EGFL7 in glioma in a Notch-dependent context it would be necessary to first understand not only the role of Notch receptors and ligands but also Notch signalling in general in malignant brain tumours. This is certainly a fascinating topic, but too complex to be addressed in the time frame of this revision and likely difficult to answer conclusively as questions concerning Notch in glioma have persisted for some time.

3) The precise mechanism by which EGFL7-mediated upregulation of $\alpha V\beta 3$ and $\alpha 5\beta 1$ regulate glioma tumour growth should be better defined.

Response to the reviewer
In order to address the reviewer's concern we analyzed Rho GTPase signaling downstream of integrins. In primary endothelial cells, EGFL7 and Fn preferentially activated Cdc42 or Rac1, respectively. In combination, both proteins annihilated each other, allowing for cell migration rather that adhesion and thereby supporting our hypothesis on EGFL7's influence on coordinated $\alpha V\beta 3/\alpha 5\beta 1$ trafficking (Fig. 4F-I). Furthermore, GL261 glioma cells ectopically expressing EGFL7 were implanted in the striatum of C57BL/6 mice, which were treated with an $\alpha 5\beta 1$ integrin-inhibiting antibody twice a week upon tumour implantation. Treatment significantly increased the survival time of the animals by 4.5 days on average (Fig. 5H).

Referee #2 (Remarks for Author):
1) The medical impact appears modest. In fact, while the data show that EGFL7 does have a role in glioma angiogenesis, its targeting is not more effective than the established (albeit still unsatisfactory) approach of VEGF blockade and their combination does not significantly improve the outcome of anti-VEGF alone. Further, the combination treatment does not significantly improve any vascular parameter compared to anti-VEGF or anti-EGFL7 alone, including vessel density, suggesting there is no additive or synergistic anti-angiogenic effect, in agreement with the modest and not-significant effect on duration of survival.

Response to the reviewer

Though we appreciate the reviewer's opinion, we are convinced that our findings have major medical relevance and that anti-EGFL7 treatment could be an alternative to anti-VEGF treatment. The reviewer's comment implies that anti-EGFL7 might be an add-on to anti-VEGF treatment only. However, as we discuss in our manuscript, the AVAglia and RTOG 0825 studies very convincingly show that anti-VEGF treatment remains palliative in glioma patients. The patients have no overall survival benefit, and at best their symptoms are slightly reduced within a narrow time window. Therefore, bevacizumab (Avastin®), the best-known VEGF-inhibitor, has not been accredited for glioma treatment by the EMA in the EU. Only the FDA approved it in the US and it is used in Switzerland as a palliative treatment. In light of these studies, it seems unlikely that anti-VEGF-treatment will remain a significant option for the treatment of malignant glioma in the EU. Other anti-angiogenesis treatments seem the logical alternative.

Instead, anti-EGFL7 treatment seems promising and increased the survival time in our glioma models comparably to anti-VEGF (Fig. 6 and Suppl. Fig. 6). Although Reviewer 2 is right that the add-on effect on animal survival reached by a combination of both treatments was not highly significant ($P = 0.17$ for GL261 and $P = 0.29$ for U87), a strong tendency towards improvement in the anti-VEGF regimen by anti-EGFL7 was observed. As a matter of fact, most vascular parameters measured displayed an additive effect upon combination therapy. The arrangement of the data in Fig. 6 and Suppl. Fig. 6 may have been misleading, so therefore we rearranged these figures for the sake of clarity. The combinatorial treatments are now shown as last data point in each line.

The additive effect of anti-EGFL7 on anti-VEGF treatment is further strengthened by our new combinatorial paradigm, where we applied both antibodies together with the chemotherapeutic agent temozolomide (new Fig. 6 K). EGFL7 significantly increased the median survival under this condition suggesting a beneficial effect of anti-EGFL7 for standard glioma therapy.

Furthermore, the EGFL7 protein holds some promising advantages over VEGF as a target molecule. EGFL7 is a secreted and barely soluble protein (comparable to fibronectin) which resides within the extracellular matrix of the tumor once the blood vessels are gone upon therapy. There it supports the regrowth of blood vessels along the trails of the former blood vessels during tumor recurrence. Thus, blocking EGFL7 slows blood vessel re-growth. In particular, this is relevant for malignant glioma as in this type of neoplasm usually the recurrence kills the patient, not the primary tumor. Therefore, anti-EGFL7 treatment does not only act comparably to anti-VEGF treatment (and likely improves it) but in addition offers some remarkable advantages over it.

In sum, anti-EGFL7 treatment might be used to reduce cytotoxicity of anti-VEGF in combinatorial regimens or it may even serve as an alternative treatment for anti-VEGF treatment itself. Therefore, we are convinced that our work is of major medical relevance for patients suffering from non-curable malignant glioma.

2) The mechanistic part, showing the effects of EGFL7 on integrin recycling and in vitro endothelial sprouting, is limited in scope. On one hand, the data provide only an incremental step in knowledge compared to previous results by the same group, and on the other they fail to address the actual relevance of these findings for glioma therapy. Does targeting of α5β1 recapitulate the effects of anti-EGFL7 treatment in experimental glioma models? Or is the effect of EGFL7 targeting in gliomas due to other EGFL7 functions that have been described, for example its regulation of Notch signaling?

Response to the reviewer

The reviewer's opinion is well taken; however, understanding how integrin αVβ3 affects intracellular trafficking of other integrins such as α5β1 is neither trivial nor irrelevant. If we want to understand why treatment with promising drugs such as the αVβ3 inhibitor cilengitide does not translate into a better prognosis or additional survival of glioma patients we need to understand how different integrins affect each other. This was in itself a challenging task and to understand in addition how EGFL7 influences this nexus is, in our view, a major step forward.

As described above in response to Reviewer 1, Point 3, in order to strengthen the understanding of the molecular mechanism behind our observations we analyzed Rho GTPase signaling downstream of integrins. These new results support our hypothesis on EGFL7's influence on coordinated

αVβ3/α5β1 trafficking (Fig. 4F-I). Furthermore, upon tumor engraftment of GL261 glioma cells ectopically expressing EGFL7, mice treated with an α5β1 integrin inhibiting antibody exhibited significantly increased (by about 4.5 d) survival times (Fig. 5H). Clearly, these data show that EGFL7 affects glioma formation dependent on both integrins αVβ3 and α5β1.

3) The proposed molecular mechanism that EGFL7 increases surface expression of α5β1 integrin through ligation with αVβ3 makes sense, but it would need to be proven by a loss-of-function experiment, showing that interference with αVβ3 by a blocking antibody or siRNA knockdown does prevent the effects of EGFL7 on α5β1.

Response to the reviewer
As suggested, we have performed additional experiments in HUVECs using either the siRNA-based knock-down of integrin αVβ3 or an αVβ3-specific blocking antibody. Interference with integrin αVβ3 reduced the EGFL7-induced upregulation of surface integrin α5β1 in primary endothelial cells. Unfortunately, the treatments themselves affected integrin α5β1 in the absence of EGFL7, thus rendering these results inconclusive. At this stage, we see no possibility to circumvent this technical problem and therefore have not incorporated this data into the manuscript.

4) The data show that EGFL7 targeting reduces vessel maturation and increases permeability, but no attempt is made to address how EGFL7 may regulate these processes.

Response to the reviewer
In order to analyze the influence of EGFL7 on vessel maturation and permeability we performed several MRI studies to measure Gadovist extravasation and stained for the recruitment of mural cells and basal membrane deposition. Further, we provided a model on the interplay between αVβ3 and α5β1 integrin to molecularly describe our observations. We now include additional molecular data on how EGFL7 modulates GTPase signaling downstream of integrins in endothelial cells (Fig. 4F-I).

Previously, the physiological role of EGFL7 for the formation of the blood-brain barrier was studied by Schmidt et al. (Development, 2007). Recently, we investigated the role of EGFL7 in pathological blood-brain barrier formation in multiples sclerosis (Larochelle et al., Nat Commun, 2018). The reviewer is right that further studies on how EGFL7 affects vessel maturation and permeability would be very interesting, but the pathological environment of malignant glioma does not seem suitable for this purpose. Preferentially, this should be done in a physiological setting using different tools and mouse lines than the ones applied in this study and is therefore beyond the scope of this manuscript.

Other points
1) Fig. 1B - It is unclear how to interpret this panel: why do red circles appear to indicate methylation in CpG island 1 and no methylation in CpG island 2?

Response to the reviewer
The red circles demarcate tumor entities that differ from the gross of the tumor samples analyzed. CpG island 1 is found mostly unmethylated except in some samples of the RTKII subgroup (red circle labeled by one star). Conversely, CpG island 2 is found mostly methylated except in the G34R subgroup (red circle labeled by two stars). This is also described in Figure legend 1B: "Methylation arrays of primary glioblastoma (GBM) specimens revealed that CpG island 1 (egfl7 promoter) was mostly unmethylated with the exception of some samples in the RTKII subgroup (*). CpG island 2 (miR-126 promoter) was found methylated in most cases except the G34R subgroup (**)."

2) Suppl. Fig. 1D - It would be useful to include a scale also in the lower part of the y axis to interpret the range of the black bars: do they reach to 1? 2? 5?

Response to the reviewer
Suppl. Fig. 1D has been changed according to the reviewer's suggestion.

3) Suppl. Fig. 1E - I could not find any method for the derivation of organotypic spheroids from patient-derived GMB biopsies.

Response to the reviewer
An appropriate paragraph on patient-derived xenografts (PDX) has been added to the materials and methods section.

4) Suppl. Fig. 1F - The EGFL7 stains are difficult to interpret, possibly due to low magnification, and also appear to delineate dot-like structures rather than vessels, like evident instead in the histochemistries shown in Fig. 1E. Higher-magnification and clearer images would be helpful.

Response to the reviewer
The figure has been improved in order to address the reviewer's concern.

5) The conclusion that EGFL7 expression in glioma specimens was restricted to blood vessels is substantiated by the data, but that it occurred independently of miR-126/126* would be strengthened by similar analysis of its pattern of expression (or lack thereof) in the patient samples by in situ hybridization.

Response to the reviewer
This is an excellent suggestion, however, the expression of EGFL7 and miR126 might be independent of each other but not necessarily exclusive. In order to address the reviewer's concern we applied two additional mouse models. First, we reduced EGFL7 expression in BTPC11 glioma cells using a shRNA-based knocked-down approach and implanted these cells into the striatum of immune-deficient mice. Second, we applied a novel EGFL7 knock-out mouse model, which we recently developed and which allows for the specific removal of EGFL7 from blood vessels in the absence of miR126 reduction (EGFL7fl/fl;Cdh5-CreERT2). These mice were intrastriatally implanted with GL261 glioma cells. Both models revealed an increase in survival of tumor-bearing mice upon the reduction or loss of EGFL7 expression (new Figs. 2G+H), strengthening our point of EGFL7 acting as an oncogene and independent of miR126.

6) Suppl. Fig. 2B, C and F - The pattern of EGFL7 appears clearly suggestive of vascular structures, although the glioma cells are responsible for its overexpression in these over-expressing tumors. Curiously, instead, the patter in panel 2E is not vascular anymore. This localization should be verified by co-staining with endothelial and glioma markers and an explanation would be useful.

Response to the reviewer
The structures staining positive for EGFL7 belong to the extracellular matrix surrounding the tumor cells. In the tumor bulk we did not observe a comparable massive amount of blood vessels, which are rather detected in the peritumoral rim of the glioma mass in our models. As a secreted protein, EGFL7 is transported via the endoplasmic reticulum and the golgi apparatus to the cellular exterior and deposited there in the extracellular matrix (Schmidt et al., Development, 2007), comparable to fibronectin. Neither GL261 nor U87 cells express endogenous EGFL7; therefore, these cells have been genetically engineered to ectopically express human or mouse EGFL7. Both proteins have been immunohistochemically stained post intracranial implantation as a proof of concept in order to validate stable EGFL7 expression during the course of the experiments. The detection of endogenous EGFL7 in the blood vessels of the mouse is notoriously difficult and works only with a few antibodies. Unfortunately, the antibodies used in this experiment were able to discriminate between recombinant human and mouse EGFL7 but did not pick up the signal of the endogenous vascular EGFL7, which remained invisible in this assay.

7) Fig. 3D-E - The provided MRI images appear to serve just a decorative function, since only one condition is shown and it is not actually mentioned which one it is. On the other hand, the graph in panel A is rather superfluous (intrastriatal injection and subsequent histological analyses are straightforward and were already described in a cartoon in Fig. 2A) and it could be removed, using the space to show complete MRI data for all conditions.

Response to the reviewer
According to the reviewer's suggestions, we included coronal MR images of all conditions (Fig. 3C+D), including T2-weighted MR images (delineation of the tumor in the right hemisphere) as well as T1-weighted images (assessment of contrast media leakage in tumors). These representative slices have been obtained from a stack of images covering the complete brain and from a larger cohort of data.

8) Figs. 2 and 5 analyze the same experiment and would best follow each other rather than being separated by the integrin data.

Response to the reviewer
The order has been switched according to the reviewer's suggestion.

9) How were histological quantifications of vascular parameters actually performed? Vessel density is presented as cubic microns, which seems more a unit of volume than of density. What are the arbitrary units used to score vascular maturation? This information is missing from the methods and quite important to understand what is being measured and its significance. In fact, for example, just looking at the immunofluorescence images in Fig. 3 and Suppl. Fig. 3, pretty much all endothelial structures appear associated with collagen IV, SMA and PDGF-Rb signals in all conditions (except the single vessel shown in the control of Fig. 3G). This would suggest that there are no "naked vessels", but then quantifications in Arbitrary Units show dramatic differences for all these parameters.

Response to the reviewer
Due to the heterogeneous vasculature in brain tumors we chose to analyze 3D image stacks, which yielded more precise quantifications as compared to 2D slices. Therefore, confocal microscopy images were acquired as stacks consisting of five confocal scans, which covered the depth of 250 μm of a histological section in total. The intensity of fluorescent staining of the endothelial cell marker CD31 was quantified by Imaris software and, due to the 3D reconstructions, calculated as the average of volume sums with the results plotted in cubic microns. Not every image may mirror the total result which emerged from a large amount of analyzed images. The pictures were intended to allow the reader to judge the staining quality and structures chosen for analysis during the experimental procedure. They have not been selected to represent the individual bars, which could only be judged on visualizing the complete 3D stack. However, in order to address the reviewer's concern images have been replaced by ones that more closely resembled the total result (Fig. 3 and Suppl. Fig 3). Vessel maturation was estimated according to the abundance of pericytes (PDGFRβ), smooth muscle cells (SMA) and basement membrane (Col IV) accompanying blood vessels, which were identified by CD31 staining. Arbitrary units represent the fluorescence intensity sums of PDGFRβ, SMA or Col IV in close proximity to CD31. We have now added this information to the material and methods section.

3rd Editorial Decision   19 June 2018

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed report from the referee asked to re-assess it. As you will see the reviewer is now globally supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

1) Please address the minor text change commented by referee 2.

Please address both referees' comments in writing. At this stage, we'd like you to discuss referee's 1 points and if you do have data at hand, we'd be happy for you to include it, however we will not ask you to provide any additional experiments at this stage.
Please provide a letter INCLUDING my comments and the reviewer's reports and your detailed responses to their comments (as Word file).

I look forward to reading a new revised version of your manuscript within 2 weeks.

***** Reviewer's comments *****

Referee #2 (Remarks for Author):

The Authors have performed several new experiments to address the weaknesses that had been identified. In particular, the new data, showing that combined anti-VEGF and anti-EGFL7 treatment significantly improves efficacy of a standard chemotherapeutic regimen, overcomes the previous

limitations in medical impact. The new experiments on the effects of integrin manipulation in vivo and their role in the mechanism of action also nicely extend and complete the extent of the findings.

Just a couple of very minor clarifications might be useful for the readers:
- The Authors mention the selection of new immunofluorescence images depicting vessel maturation in Figure 3, to better reflect the quantified values in the graphs below. However, it appears to me that the new Figure file still contains the same images as the first version.

- In the new panel 6K, it would be useful to explicitly clarify, both in the legend and the results text, that chemotherapy is given in all conditions as a baseline, and that therefore "control" actually means chemotherapy alone.

3rd Revision - authors' response   02 July 2018

Editor (Remarks for Author):
1) Please address the minor text change commented by referee 2. Please address both referees' comments in writing. At this stage, we'd like you to discuss referee's 1 points and if you do have data at hand, we'd be happy for you to include it, however we will not ask you to provide any additional experiments at this stage. Please provide a letter INCLUDING my comments and the reviewer's reports and your detailed responses to their comments (as Word file).

Response to the editor
Please find the discussion of the reviewer's points below.

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Response to the reviewer
These changes affected the control panels in Fig. 3G+I. However, once again we'd like to point out that these are just representative pictures to allow for judging on staining quality and structures chosen for subsequent analysis. Quantifications illustrated in the corresponding graphs have been made by usage of 3D stacks.

In the new panel 6K, it would be useful to explicitly clarify, both in the legend and the results text, that chemotherapy is given in all conditions as a baseline, and that therefore "control" actually means chemotherapy alone.

Response to the reviewer
The term "control" in Figure 6 has been substituted by "TMD", the abbreviation for temozolomide, as suggested by the reviewer.

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Mirko HH Schmidt
Journal Submitted to: EMBO Molecular Medicine
Manuscript Number: EMM-2017-08420-V3

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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

A- Figures
1. Data
The data shown in figures should satisfy the following conditions:

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

2. Captions

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

→ a specification of the experimental system investigated (eg cell line, species name).
→ the assay(s) and method(s) used to carry out the reported observations and measurements
→ an explicit mention of the biological and chemical entity(ies) that are being measured.
→ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

→ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
→ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
→ a statement of how many times the experiment shown was independently replicated in the laboratory.
→ definitions of statistical methods and measures:
  • common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  • are tests one-sided or two-sided?
  • are there adjustments for multiple comparisons?
  • exact statistical test results, e.g., P values = x but not P values < x;
  • definition of 'center values' as median or average;
  • definition of error bars as s.d. or s.e.m.

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

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size calculations were not performed. However, based on the preliminary data and more importantly on the consistency and effective differences we measured between the experimental groups, sample size was determined to be adequate.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	See method section
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	In all animal experiments, survival time was monitored from the moment the animals woke up from narcosis, and remarked as Day 1. Thus, no exclusion criteria were used.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	All experimental animals were of the same genetic background and from the same strain, same age (within the week), same gender, same fur colour, similar weight and comparble health status. Animals were, depending on the experiment, simply randomized into two, three or four groups. The exception were the experiments which results were depicted in the Figure 2E, F and H, where the survival of the animals was compared between KO and WT littermates. Here, no randomization was applicable.
For animal studies, include a statement about randomization even if no randomization was used.	According to the best experimental practice, the animals used in all animal experiments, with the exception of three experiments with KO animals (Figure 2E, G and H) were of the apperiance, genetic background and health status. Thus, the simple randomization was used.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No blinding was used.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Experimentators were not blinded to the treatment groups.
5. For every figure, are statistical tests justified as appropriate?	Yes. Statistical analysis was performed using one-way analysis of variance (ANOVA) or in case of comparing two groups Mann-Whitney U-test. For the comparison of survival curves the log-rank test was used. All test are appropriate for analyses of differences in the expression levels, tumore sizes, blood vessels maturation parameters or survivals between different group of animals. This is precisaly stated in the mansucript and figure legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Samples were tested by GraphPad Prism Shapiro-Wilk normality test for normal distribution.
Is there an estimate of variation within each group of data?	Yes

Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	See method section
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	See method section. Cell lines are routinely tested for mycoplasma contamination.

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	All the animals used in the study are stated in the manuscript, with the details regarding source, species, strain, gender, age and housing and husbandry conditions.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal experiments were approved by the ethics committee of the Landesuntersuchungsamt Rheinland-Pfalz, Germany and conducted according to the German Animal Protection Law §8 Abs. 1 TierSchG. See method section.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	I am authorised to confirm compliance with the ARRIVE Guidelines on behalf of my competent authority.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	The use of tumor tissue was approved by the ethical committee of the Goethe University Hospital (GS04/09). See method section.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	The auhtors confirm that informed consent was obtained from all subjects and experiments conformed to the principles set out in the declaration of Helsinki and the Department of Health and Human Services Belmont Report.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

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