

Peer Review Information

Journal: Nature Cell Biology

Manuscript Title: EGFR ligand shifts the role of EGFR from oncogene to tumor suppressor in EGFR amplified glioblastoma by suppressing invasion through BIN3 upregulation

Corresponding author name: Aryn A. Habib

Reviewer Comments & Decisions:

Decision Letter, initial version:

Subject: Decision on Nature Cell Biology submission NCB-H46222

Message:

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Dear Dr Habib,

Your manuscript, "EGFR ligand shifts the role of EGFR from oncogene to tumor suppressor in EGFR amplified glioblastoma", has now been seen by 3 referees, who are experts in EGFR biology and brain tumor. As you will see from their comments (attached below) they find this work of potential interest, but have raised substantial concerns, which in our view would need to be addressed with considerable revisions before we can consider publication in Nature Cell Biology.

Nature Cell Biology editors discuss the referee reports in detail within the editorial team, including the chief editor, to identify key referee points that should be addressed with priority, and requests that are overruled as being beyond the scope of the current study. To guide the scope of the revisions, I have listed these points below. We are committed to providing a fair and constructive peer-review process, so please feel free to contact me if you would like to discuss any of the referee comments further.

In particular, it would be essential to:

A) Address the confounding effect of the cell culture system as questioned by Reviewer 1;

"One critical issue that compromises the interpretation of many (if not most) of the data obtained throughout the study is the conditions under which the PDX cells are cultured *ex vivo*. It has been previously demonstrated (and has been in fact referenced by the authors themselves) that EGFR amplification is lost in GBM cells grown in culture. In fact, it is now well established that the presence of serum in the media drives the loss of the EGFR amplicon (PMID: 20803305, 14603439, 16697959). Therefore, considering that some of the cells used in the study were kept in culture long enough to undergo antibiotic selection and expansion, it is very conceivable (and possibly likely) that the levels of EGFR in those cells, compared to the parental population (i.e. when first harvested from the PDX) has

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changed. Given the importance of EGFR levels in the interpretation of the data, and given the fact that a significant portion of the mechanistic experiments were done with these cells, this issue is of particular relevance. Levels of EGFR should have been documented over time. Alternatively, these cells could have been grown in stem cell conditions as were the patient-derived neurosphere lines."

B) Strengthen the evidence to support the proposed mechanism;

Reviewer 1

"In figure 2I, the increase in BIN3 levels in DOCK7 immunoprecipitates from EGF-treated cells tracks with the increase in BIN3 levels seen in whole cell lysates. This might suggest that the association between BIN3 and DOCK7 is constitutive and therefore primarily regulated by protein expression levels. However, in figure 2J, the levels of DOCK7 associated with BIN3 immunoprecipitates seem to increase following EGF treatment despite unchanged levels of DOCK7 in whole cell lysates, and similar levels of BIN3 in all immunoprecipitates. This would instead indicate that the BIN3/DOCK7 interaction might be regulated not only by BIN3 levels, but also by other EGF-dependent events. The authors need to explain this."

"Figure 4H shows no interaction between EGFR and TAB1 in parental GBM12 cells despite the decrease in invasion seen with EGFR siRNA in these cells (Figures 1C and 1G), and the observed interaction when assessed by M/S (Figure 4G). How do you reconcile the fact that EGFR siRNA inhibits invasion, but TAB1 siRNA does not?"

Reviewer 3

"The most critical concern of mine is that how can we define constitutive EGFR signaling? It was not clearly described in the paper. Was it decided by the level of EGFR only? If so, Knockdown of EGFR make GBMs from constitutive to ligand signaling, and overexpress of EGFR shifts GBMs from ligand inducing signaling to constitutive signaling. In EGFR knockdown cells, treatment of EGF will enhance the invasion just like GBM622 while your results indicated that the invasion percentage rarely changed in Figure 1E. If it still harbors the character of constitutive signaling. It's obvious not acceptable. If it was decided by the reaction to EGF, it was too subjective to measure by objective experiments."

"The mechanism remains largely unknown, the main theme of this paper should focus on the different status of EGFR signaling in constitutive signaling or ligand induced signaling such as the different phosphorylation site or spatial change, and the downstream cascade. If EGFR signaling status was not changed, why different downstream cascade happened? If the mechanism was still unclear and we thought that EGF affect other unknown receptors and EGFR signaling was not a dominate."

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"There were some contradictions in Figure3Q-S. BIN3 was claimed as a tumor suppressor. Why knocking down of BIN3 had no influence on invasion percentage and the overall survival of mouse?"

C) All other referee concerns pertaining to strengthening existing data, providing controls, methodological details, clarifications and textual changes as applicable should also be addressed.

E) Finally please pay close attention to our guidelines on statistical and methodological reporting (listed below) as failure to do so may delay the reconsideration of the revised manuscript. In particular please provide:

- a Supplementary Figure including unprocessed images of all gels/blots in the form of a multi-page pdf file. Please ensure that blots/gels are labeled and the sections presented in the figures are clearly indicated.

- a Supplementary Table including all numerical source data in Excel format, with data for different figures provided as different sheets within a single Excel file. The file should include source data giving rise to graphical representations and statistical descriptions in the paper and for all instances where the figures present representative experiments of multiple independent repeats, the source data of all repeats should be provided.

We would be happy to consider a revised manuscript that would satisfactorily address these points, unless a similar paper is published elsewhere, or is accepted for publication in Nature Cell Biology in the meantime.

When revising the manuscript please:

- ensure that it conforms to our format instructions and publication policies (see below and <https://www.nature.com/nature/for-authors>).

- provide a point-by-point rebuttal to the full referee reports verbatim, as provided at the end of this letter.

- provide the completed Reporting Summary (found here <https://www.nature.com/documents/nr-reporting-summary.pdf>). This is essential for reconsideration of the manuscript will be available to editors and referees in the event of peer review. For more information see <http://www.nature.com/authors/policies/availability.html> or contact me.



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- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

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Please submit the revised manuscript files and the point-by-point rebuttal to the referee comments using this link:

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We would like to receive a revised submission within six months.

We hope that you will find our referees' comments, and editorial guidance helpful. Please do not hesitate to contact me if there is anything you would like to discuss.

Best wishes,

Zhe Wang

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Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

The manuscript by Guo et al describes an interesting and extensive set of experiments set out to investigate the molecular basis for the opposing biological outputs of ligand-induced and ligand-independent effects of EGFR on oncogenic phenotypes in glioblastoma. The authors demonstrate that EGFR ligand stimulation can suppress invasion in several in vitro GBM models, and that ectopic expression of TGF α , while increasing cell proliferation, reduces tumour burden and confers improved survival in intracranial GBM xenografts in vivo. The authors present a variety of in vitro and in vivo data that link this reported EGF-induced suppression of invasion to the EGR1-mediated induction of BIN3, which is proposed to work by directly binding to DOCK7 and inhibiting its RhoA-activating function. Furthermore, the authors present some evidence that the specific effects of EGF on invasion are



dependent on the expression levels of EGFR, and that (based on analysis of TCGA data) EGFR ligands are oncogenic in EGFR-non-amplified GBMs but tumour suppressive in EGFR-amplified tumours.

The discovery of a tumour suppressive function for EGFR in glioblastoma, where EGFR activating lesions are found in more than half of all tumours, is of critical importance and could have enormous therapeutic implications. Therefore, the findings in this study, if adequately vetted, would represent an important advance in the field. However, the study falls short of convincingly demonstrating that EGFR levels are the molecular toggle that determines the direction of the EGF-induced phenotype. One critical issue that compromises the interpretation of many (if not most) of the data obtained throughout the study is the conditions under which the PDX cells are cultured *ex vivo*. It has been previously demonstrated (and has been in fact referenced by the authors themselves) that EGFR amplification is lost in GBM cells grown in culture. In fact, it is now well established that the presence of serum in the media drives the loss of the EGFR amplicon (PMID: 20803305, 14603439, 16697959). Therefore, considering that some of the cells used in the study were kept in culture long enough to undergo antibiotic selection and expansion, it is very conceivable (and possibly likely) that the levels of EGFR in those cells, compared to the parental population (i.e. when first harvested from the PDX) has changed. Given the importance of EGFR levels in the interpretation of the data, and given the fact that a significant portion of the mechanistic experiments were done with these cells, this issue is of particular relevance. Levels of EGFR should have been documented over time. Alternatively, these cells could have been grown in stem cell conditions as were the patient-derived neurosphere lines. Secondly, the role of EGFRvIII in determining the influence of EGF on invasion is inadequately explored. Both EGFRvIII models used in the study appear to express mainly the mutant form (hard to see ANY wild type EGFR in the blots shown in Figure 1A, lanes 3 and 8), with only a very small amount of wild type receptor (GBM6 and GBM9). It would be important to determine the allelic frequency of both WT and vIII in these models, Stoichiometrically speaking, it seems the majority of dimers (or higher-order oligomers) will contain EGFRvIII only, and these would signal completely independently of ligand.

Third, there is, as the authors themselves have pointed out, evidence from other groups demonstrating a pro-invasion effect for EGFR ligands in some experimental models. The authors claim that this can be explained by EGFR expression levels being low in established GBM cell lines, and provide evidence that through EGFR overexpression in U251 cells EGF turns from being pro-invasive to anti-invasive. However, there are other studies that show this not to be the case. For example, Lund-Johansen et al (PMID: 1523973) showed that EGF induces invasion/migration in GBM cells with low, medium, and high levels of EGFR expression (as measured by EGF binding, PMID: 2253244). The same study demonstrated that Ab-528, an anti EGFR mAb that blocks EGF binding inhibited invasion/migration. Similarly, Tony Hunter's group showed many years ago (PMID: 11359909) that in cell lines with high level EGFR overexpression,



EGF induced cell migration/invasion through inactivation of FAK. Are the authors proposing that this particular mechanism is not functional in GBM cells? Perhaps FAK activation should have been examined. Finally, a study by Stec et al (PMID: 27004406) using the GBM cell line DK-MG, showed that when cells were sorted into high and low-EGFR expressing populations, EGF induced invasion in the former and had no effect on the latter. These studies need to be acknowledged, at the very least. But, more importantly, they raise the possibility that an additional layer of regulation as yet to be identified acts as the toggle that switches pro-invasion into anti-invasion EGFR-dependent functions, and that ligand binding or EGFR expression levels cannot alone explain these differences.

There are also some additional issues, some of which have to do with inconsistencies in the data, or limited interpretation of the data including the following:

1. In p19 the authors say, “These data indicate that constitutive EGFR activity drives invasion while ligand-activated EGFR activity suppresses invasion.” What is the evidence that EGFR is constitutively active in these examples (1K-L)? Given that supplementation of EGF in cells with ectopically overexpressed EGFR leads to a level of invasion similar to untreated parental cells, an alternative interpretation is that given sufficient EGFR protein, EGF has no biologically meaningful effects on invasion. In other words, in the presence of sufficient EGF, EGFR expression levels seem irrelevant to invasion capacity (Supplemental 2D, e.g.)
2. In Figure 1N, EGFR is not at all overexpressed in GBM9 cells transduced with the WT EGFR construct, but a significant increase in cell invasion is still observed. In fact, there may even be a decrease in EGFR in the transduced line.
3. The authors argue that “ligand-activated EGFR activity suppresses invasion.” However, the cetuximab experiments show that blocking ligand binding does not affect invasion. This would assume that there is no ligand-bound EGFR under the steady-state conditions of growth under which the invasion assays are carried out. This is simply not true. In fact, the western blot shown in Supplementary Figure 1N shows that in the absence of added EGF, EGFR phosphorylation goes down with cetuximab treatment (lanes 1 vs 3). Also, the authors failed to show that pre-treating cells with cetuximab prevented EGF-induced suppression of cell invasion.
4. Although they show that GBM14 cells ectopically expressing EGFRvIII have enhanced invasion, they do not show whether EGF is ineffective at blocking invasion in these cells.
5. To make any claims about U251 EGFR levels being low, they need to run them alongside their PDX-derived and neurosphere cells for comparison. GSC11 cells, for example, have “low” EGFR levels but invasion is also inhibited by EGF in these cells.



6. Others have previously shown that BIN3 depletion increases invasion (PMID: 18339847). In fact, Fig 1R could be read as “in the presence of EGF, loss of BIN3 increases cell invasion.”
7. It is unclear why the addition of EGF does not inhibit invasion in cells that overexpress DOCK7 (Supplementary 2R) despite EGF still being able to suppress RhoA activation (Supplementary 2S).
8. In figure 2I, the increase in BIN3 levels in DOCK7 immunoprecipitates from EGF-treated cells tracks with the increase in BIN3 levels seen in whole cell lysates. This might suggest that the association between BIN3 and DOCK7 is constitutive and therefore primarily regulated by protein expression levels. However, in figure 2J, the levels of DOCK7 associated with BIN3 immunoprecipitates seem to increase following EGF treatment despite unchanged levels of DOCK7 in whole cell lysates, and similar levels of BIN3 in all immunoprecipitates. This would instead indicate that the BIN3/DOCK7 interaction might be regulated not only by BIN3 levels, but also by other EGF-dependent events. The authors need to explain this.
9. Figure 4H shows no interaction between EGFR and TAB1 in parental GBM12 cells despite the decrease in invasion seen with EGFR siRNA in these cells (Figures 1C and 1G), and the observed interaction when assessed by M/S (Figure 4G). How do you reconcile the fact that EGFR siRNA inhibits invasion, but TAB1 siRNA does not?
10. Given that EGFR amplification is a feature of the classical transcriptional subtype (which has distinct biology), is it not possible that the prognostic value of EGFR ligands is linked to the transcriptional subtype rather than EGFR levels? Can this be evaluated?
11. In p33 the authors state, “Although constitutively active mutants are expressed in GBM, the most common EGFR type expressed in GBM is EGFRwt. While expression of EGFRwt is associated with a low level of EGFR phosphorylation, addition of EGFR ligand results in a much greater phosphorylation of the EGFR (Supplementary Fig. 5I), suggesting that overall EGFR phosphorylation in GBM is likely to largely reflect ligand-activated EGFRwt.” While this is true, the frequency of EGFR mutation (including high level focal amplification which leads to ligand-independent activation) is high enough that it could drive the enrichment.

Minor points:

1. GBM14 should have been included as a negative control in Figure 1O (i.e. in response to EGF).
2. Figure 3Q is missing statistics
3. Figure 5K, increase in pEGFR following STAT3 KD is marginal, despite significant increase in BIN34
4. In Figure 6R, should you not expect that knockdown of HB-EGF increase invasion?



5. Although the effects of erlotinib were measured at both 24 and 48hrs, the effects of EGF stimulation were only assessed at 24hrs throughout the manuscript.

6. Authors state, "A time course of the erlotinib effect and its impact on signaling is shown in Supplementary Fig. 1J-L." Supplementary Figure 1 does not show any time-course signalling data following erlotinib treatment. Also, there are only two time points which barely qualifies as a time course.

For all these reasons, I cannot recommend publication of this manuscript in Nature Cell Biology unless all of these points have been satisfactorily addressed.

Reviewer #2:

Remarks to the Author:

This is a complex and dense paper by a thought-leader in the field, that makes a number of new and important insights into EGFR signaling in glioblastoma, examining the apparent paradoxical effects of ligand binding in blocking invasion while promoting proliferation. While one would anticipate that ligand binding would thus lead to a contest, balancing increased proliferation against decreased invasion, experiments proposed show that tumors are both smaller and less invasive (despite showing higher levels of proliferation) a paradox that is not explained.

While experiments in this paper are done to a high standard, and are generally convincing, the stories here are both dense and complicated. The authors might consider stripping the invasion/proliferation story to make this a simpler tale (Figs 1-3 and 7) and then writing a second paper that incorporates details and translational importance from Figs 4-6.

Other thoughts:

1. Authors state that, "the level of EGFR expression...determines whether the outcome of ligand-dependent EGFR signaling is increased or decreased invasion". Can this result (that ligand activation activates invasion in cells with low EGFR) be validated in additional EGFR-low GBM lines? In this regard, why don't cells in Figs 1C-1F show increased invasion in response to siEGFR + EGF?

2. In the setting of erlotinib treatment over time (Fig S1L), does erlotinib lead to reduced expression of BIN3, and do cells that recover from erlotinib induced suppression of invasiveness show re-expression of BIN3?

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3. In Fig 4, how do authors reconcile apparently conflicting results of higher proliferation resulting in smaller tumors?
4. IP in Fig 4H shows very poorly focused EGFR band, can this be repeated?
5. Early figures in the manuscript suggest that Dock7 is the major driver of invasion, and later figures demonstrate the importance of EMP1. Are these parallel or intersecting pathways that promote invasion?

Reviewer #3:

Remarks to the Author:

In the paper “EGFR ligand shifts the role of EGFR from oncogene to tumor suppressor in EGFR amplified glioblastoma” Guo, et al claimed that in EGFR amplified GBMs EGF inhibited the invasion of GBMs. The results were of interest and the conclusion was pretty challenging, however, there were some major issues which need to be very clearly addressed, to support the main conclusion.

Major:

1. The most critical concern of mine is that how can we define constitute EGFR signaling? It was not clearly described in the paper. Was it decided by the level of EGFR only? If so, Knockdown of EGFR make GBMs from constitute to ligand signaling, and overexpress of EGFR shifts GBMS form ligand inducing signaling to constitute signaling. In EGFR knockdown cells, treatment of EGF will enhance the invasion just like GBM622 while your results indicated that the invasion percentage rarely changed in Figure 1E. If it still harbors the character of constitute signaling. It's obvious not acceptable. If it was decided by the reaction to EGF, it was too subjective to measure by objective experiments.
2. How to evaluate different invasion status regarding the level of p-EGFR? As you mentioned in the paper, constitute or ligand induced EGFR signaling both promotes the phosphorylation of EGFR but resulted in different results.
3. The mechanism remains largely unknown, the main theme of this paper should focus on the different status of EGFR signaling in constitute signaling or ligand induced signaling such as the different phosphorylation site or spatial change, and the downstream cascade. If EGFR signaling status was not changed, why different downstream cascade happened? If the mechanism was still unclear and we thought that EGF affect other unknown receptors and EGFR signaling was not a dominate.
4. The microarray you used in ref 2 was not suitable here. You compare EGFR-low cells with EGFR-low + EGFR cells. This was not correct because the genetic background of EGFR-low cells and EGFR amplified

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cells were different. In this paper, you should collect GBMs (GBM6 GBM12) treated with or without EGF and subjected to RNA sequence. Thus, the selection of BIN3 was unconvincing. Moreover, the microarray data was applied in U251MG, which was not acceptable in this paper, GSC should be applied instead. Your description “We found that 93 genes were upregulated by EGFR overexpression in glioma cells in the absence of exogenous EGF while 66 genes were upregulated only when EGF was added” didn’t match with the real sequence strategy as well.

5. There were some contradictions in Figure3Q-S. BIN3 was claimed as a tumor suppressor. Why knocking down of BIN3 had no influence on invasion percentage and the overall survival of mouse?
6. In Figure5A-C, tof should be applied in the presence of EGF. The proliferation rate of GBMs treated with Tof should be provided to evaluate its’ capacity as a potential drug.
7. The author claimed that “All of these studies have used established GBM cell lines that are no longer considered to be a representative model of GBM, in part because they lose the EGFR amplification” how can you ensure that your GSCs don’t lose the EGFR amplification? I mean, how if GBM6 GBM12 lose EGFR amplification, at least part of amplification compared with the GBM species.
8. The title was not appropriate as the authors claimed that treated with EGF in constitute EGFR cells promotes the proliferation. This was also an oncogene character.
9. The author claimed that GBM6 was WT+VIII, WT was almost undetectable in Figure1A. But extremely strong expressed in Figure1D. Please explain. Uncut raw data should be provided.

Minor:

10. In Figure1H. cells were transfected with siEGFR, but the immunoblot of EGFR indicated that EGFR was not knocked down. IB should be re applied to separate EGFR and EGFRVIII.
11. In Figure 3C, the slice was not stained with HE. Please check.
12. Figure 3 should be moved to the supplementary figure and the data about EGF in vivo should be moved to the main Figure.
13. There were typos such as TFG and GCS. please check and correct.
14. Figure3J-M should be moved to the supplementary figure.
15. Figure 3M,3P was mislabeled.
16. How do the authors identify NT region in GBM12B HE slice in Figure3R?
17. In Figure4A, IB should be re-applied to clearly separate EGFR and VIII.
18. Figure4I,4H need to be re-applied in the presence of EGF.
19. There were some contradictions in Figure4 D,4I,4L. knockdown of EMP1 and TAB1 rarely affected the invasion, please explain.
20. In Figure4J, TAB1 decreased in GBM12 but increased in GBM9. Please explain. IB need to be redone to separate EGFR and EGFRVIII.
21. The time point should be provided in Figure5F as it was contradictory to the results in Figure1I.

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22. What's the advantage of Tof? EGF was reported to inhibit the invasion as you mentioned previously. Why don't use EGF immediately?
23. How do you suggest to culture GSCs as additional EGF will inhibit invasion?
24. The length of the article should be reduced to meet Nature cell biology demands. The data quality should be improved especially the IB assay and the raw data should be provided as well.

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READABILITY OF MANUSCRIPTS – Nature Cell Biology is read by cell biologists from diverse backgrounds, many of whom are not native English speakers. Authors should aim to communicate their findings clearly, explaining technical jargon that might be unfamiliar to non-specialists, and avoiding non-standard abbreviations. Titles and abstracts should concisely communicate the main findings of the study, and the background, rationale, results and conclusions should be clearly explained in the manuscript in a manner accessible to a broad cell biology audience. Nature Cell Biology uses British spelling.

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AUTHOR CONTRIBUTIONS – must be included after the Acknowledgements, detailing the contributions of each author to the paper (e.g. experimental work, project planning, data analysis etc.). Each author should be listed by his/her initials.

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Methods should be written concisely, but should contain all elements necessary to allow interpretation and replication of the results. As a guideline, Methods sections typically do not exceed 3,000 words. The Methods should be divided into subsections listing reagents and techniques. When citing previous methods, accurate references should be provided and any alterations should be noted. Information must be provided about: antibody dilutions, company names, catalogue numbers and clone numbers for monoclonal antibodies; sequences of RNAi and cDNA probes/primers or company names and catalogue numbers if reagents are commercial; cell line names, sources and information on cell line identity and authentication. Animal studies and experiments involving human subjects must be reported in detail, identifying the committees approving the protocols. For studies involving human subjects/samples, a statement must be included confirming that informed consent was obtained. Statistical analyses and information on the reproducibility of experimental results should be provided in a section titled "Statistics and Reproducibility".

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- At a minimum, please include a statement confirming that all relevant data are available from the authors, and/or are included with the manuscript (e.g. as source data or supplementary information), listing which data are included (e.g. by figure panels and data types) and mentioning any restrictions on availability.



- If a dataset has a Digital Object Identifier (DOI) as its unique identifier, we strongly encourage including this in the Reference list and citing the dataset in the Methods.

We recommend that you upload the step-by-step protocols used in this manuscript to the Protocol Exchange. More details can found at www.nature.com/protocolexchange/about.

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Unprocessed scans of all key data generated through electrophoretic separation techniques need to be presented in a supplementary figure that should be labelled and numbered as the final supplementary figure, and should be mentioned in every relevant figure legend. This figure does not count towards the total number of figures and is the only figure that can be displayed over multiple pages, but should be provided as a single file, in PDF or TIFF format. Data in this figure can be displayed in a relatively informal style, but size markers and the figures panels corresponding to the presented data must be indicated.

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Each Supplementary Figure should be provided as a single page and as an individual file in one of our accepted figure formats and should be presented according to our figure guidelines (see above). Supplementary Tables should be provided as individual Excel files. Supplementary Videos should be



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Information on how many times each experiment was repeated independently with similar results needs to be provided in the legends and/or Methods for all experiments, and in particular wherever representative experiments are shown.

We strongly recommend the presentation of source data for graphical and statistical analyses as a separate Supplementary Table, and request that source data for all independent repeats are provided when representative experiments of multiple independent repeats, or averages of two independent experiments are presented. This supplementary table should be in Excel format, with data for different figures provided as different sheets within a single Excel file. It should be labelled and numbered as one of the supplementary tables, titled “Statistics Source Data”, and mentioned in all relevant figure legends.



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Author Rebuttal to Initial comments

Reviewer #1:

Remarks to the Author:

General Comment 1a:

The manuscript by Guo et al describes an interesting and extensive set of experiments set out to investigate the molecular basis for the opposing biological outputs of ligand-induced and ligand-independent effects of EGFR on oncogenic phenotypes in glioblastoma. The authors demonstrate that EGFR ligand stimulation can suppress invasion in several in vitro GBM models, and that ectopic expression of TGF α , while increasing cell proliferation, reduces tumour burden and confers improved survival in intracranial GBM xenografts in vivo. The authors present a variety of in vitro and in vivo data that link this reported EGF-induced suppression of invasion to the EGR1-mediated induction of BIN3, which is proposed to work by directly binding to DOCK7 and inhibiting its RhoA-activating function. Furthermore, the authors present some evidence that the specific effects of EGF on invasion are dependent on the expression levels of EGFR, and that (based on analysis of TCGA data) EGFR ligands are oncogenic in EGFR-non-amplified GBMs but tumour suppressive in EGFR-amplified tumours.

The discovery of a tumour suppressive function for EGFR in glioblastoma, where EGFR activating lesions are found in more than half of all tumours, is of critical importance and could have enormous therapeutic implications. Therefore, the findings in this study, if adequately vetted, would represent an important advance in the field.

Response: We appreciate the Reviewer's comment about the potentially high importance of this study. Thank you. We have tried our best to address all of the issues raised by Reviewer 1.

General Comment 1b:

However, the study falls short of convincingly demonstrating that EGFR levels are the molecular toggle that determines the direction of the EGF-induced phenotype. One critical issue that compromises the interpretation of many (if not most) of the data obtained throughout the study is the conditions under which the PDX cells are cultured ex vivo. It has been previously demonstrated (and has been in fact referenced by the authors themselves) that EGFR amplification is lost in GBM cells grown in culture. In fact, it is now well established that the

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presence of serum in the media drives the loss of the EGFR amplicon (PMID: 20803305, 14603439, 16697959). Therefore, considering that some of the cells used in the study were kept in culture long enough to undergo antibiotic selection and expansion, it is very conceivable (and possibly likely) that the levels of EGFR in those cells, compared to the parental population (i.e. when first harvested from the PDX) has changed. Given the importance of EGFR levels in the interpretation of the data, and given the fact that a significant portion of the mechanistic experiments were done with these cells, this issue is of particular relevance. **Levels of EGFR should have been documented over time.** Alternatively, these cells could have been grown in stem cell conditions as were the patient-derived neurosphere lines.

Response

We acknowledge the importance of this question. To address whether the EGFR amplification and level of EGFR expression in the Mayo PDX lines changes over time we undertook the following experiments.

The Mayo PDX model involves implantation of resected GBMs in mice where they grow as subcutaneous tumors¹. The tumors are harvested and cell cultures are generated for short term use. In those cases in which we stably transfect these cultures, they are in culture longer, for antibiotic selection and expansion of clones. To determine whether EGFR amplification is lost or EGFR levels change in these lines, we undertook the following experiments.

1. Since a key issue is whether EGFR amplification and EGFR levels are maintained in stably transfected PDX lines, we compared the EGFR level and EGFR copy number in GBM12 and GBM6 cells stably transfected with TGF α and BIN3 (Supplementary Fig 4B-C). We compared the EGFR levels in freshly harvested cells to cells stably transfected with empty vector, TGF α or BIN3. EGFR levels are quite similar (Supplementary Fig. 4B-C). Taqman qPCR was done to detect EGFR amplification²⁻⁷ and demonstrated no loss of EGFR copy number in stably transfected cells (Supplementary Fig. 4C).
2. We also compared the EGFR levels in orthotopic tumors. We compared tumors generated by injecting fresh GBM12 (or GBM6) cultures to tumors formed by injecting stably transfected Mayo PDX GBM12-TGF α or GBM12 BIN3 cultures. We found that EGFR levels in these tumors are quite similar (Supplementary Fig. 4L) This is also true for EGFR copy number (Supplementary Fig. 4M) demonstrating that the EGFR amplification is retained in tumors derived from stably transfected lines compared to tumors generated from fresh cultures.



3. Finally, Mayo PDX lines were cultured in serum containing medium for 0-8 weeks. We compared the level of EGFR expression by Western blot and EGFR copy number by Taqman qPCR. We found that EGFR level in cultures that have been in serum containing medium for 8 weeks are similar compared to EGFR levels from freshly harvested tumor cultures, when tested by Western blot (Supplementary Fig. 4D). When we looked at EGFR copy number, again we found very little change (Supplementary Fig. 4E). Also, after being cultured for eight weeks in serum containing medium, the cells continue to respond to EGFR stimulation with suppression of invasion, consistent with the stable EGFR levels Supplementary Fig. 4F

The experiments outlined above indicate that the EGFR levels in Mayo PDX cultures remain stable for the duration of our experiments. It is possible that the increased stability of EGFR in this cell culture model results from the initial passage in mice. Alternatively, and perhaps more likely, the duration of culture in serum containing medium is important. Previous studies (PMID: 20803305 cited by Reviewer 1)⁸ distinguish between short term cultures (less than 90 days) and long term cultures (greater than 90 days), and suggest that the EGFR amplification, at least in some lines, is lost in long term cultures.

Our stable transfection and expansion of cultures generally take less than 2 months. Our new data indicate that the EGFR amplification is retained in the stably transfected Mayo PDX lines, and in the tumors that are derived from stably transfected Mayo PDX lines.

It should be noted that in this study we have also used a neurosphere model that is never cultured in serum. Almost every experiment in our paper is also conducted on primary GBM cultures maintained as neurospheres and cultured in stem cell medium and are known to more closely mirror the phenotype and genotype of primary tumors including the EGFR amplification⁹.

Our results in Mayo PDXs and in neurospheres are very similar.

General Comment 2

Secondly, the role of EGFRvIII in determining the influence of EGF on invasion is inadequately explored. Both EGFRvIII models used in the study appear to express mainly the mutant form (hard to see ANY wild type EGFR in the blots shown in Figure 1A, lanes 3 and 8), with only a very small amount of wild type receptor (GBM6 and GBM9). It would be important to determine the allelic frequency of both WT and vIII in these models, Stoichiometrically speaking, it seems the majority of dimers (or higher-order oligomers) will contain EGFRvIII only, and these would signal completely independently of ligand.



Response: We have rerun multiple western blots to show the expression of EGFRwt and EGFRvIII and labeled both signals clearly (for example Fig. 4A). While it is quite true that both GBM6 and GBM9 have much more EGFRvIII than EGFRwt and one would expect these lines stoichiometrically speaking to behave as if EGFR signaling were constitutive even in the presence of ligand (since EGFRvIII is unable to bind ligand), in reality we consistently see a predominantly ligand-dependent signaling in GBM6 and GBM9 when exposed to EGFR ligand. Thus the invasive behavior of these cells upon exposure to EGF is similar to GBM12 which expresses only EGFRwt. Thus, GBM6 and GBM9 respond to EGF with suppression of invasion (Fig. 1B) upregulation of EGR1 (Fig. 2D), and upregulation of BIN3 (Fig. 1P).

The allelic frequency of EGFRwt and EGFRvIII in GBM6 is 0.066 and 0.934, and GBM9 is 0.083 and 0.917. This was determined as follows:

Allele frequencies of EGFRwt and EGFRvIII were determined by WES. Genomic DNA extracted from GBM6 and GBM9 was subjected to library preparation using the SureSelect V6 kit (Agilent) following the manufacturer's instructions. Sequencing was performed on a NextSeq500 with 150 bases of paired-end reads to target 20X of raw depth. BWA-MEM¹ was used to align sequence reads to reference genome GRCh37 (decoy). Post-BAM processing was performed using Picardtools (v2.25.5; <https://broadinstitute.github.io/picard/>), Samtools v1.12², bedtools v2.30.0³ and GATK v3.8⁴. Variants were detected using GATK v3.8⁴, Samtools v1.12², and annotation using WGSAnnot was done using WGSAnnot (whole genome sequencing annotator)⁵. From the mapping results the AFs were calculated as follows: AF of EGFRwt=The average coverage for exons 2 to 7/The average coverage for exons 1 and 8 to 28. AF of EGFRvIII=1-AF of EGFRwt. The following are references for this methodology.

1. Li H. (2013) Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. arXiv:1303.3997v1 [q-bio.GN]
2. Li H A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. *Bioinformatics*. 2011 Nov 1; 27(21):2987-93. Epub 2011 Sep 8. [PMID: 21903627]
3. Poplin R, Ruano-Rubio V, DePristo MA, Fennell TJ, Carneiro MO, Van der Auwera GA, Kling DE, Gauthier LD, Levy-Moonshine A, Roazen D, Shakir K, Thibault J, Chandran S, Whelan C, Lek M, Gabriel S, Daly MJ, Neale B, MacArthur DG, Banks E. (2017). Scaling



accurate genetic variant discovery to tens of thousands of samples bioRxiv, 201178. DOI: 10.1101/201178

4. Liu X, White S, Peng B, et al. WGS: an annotation pipeline for human genome sequencing studies. *J Med Genet*. 2016;53(2):111-112. doi:10.1136/jmedgenet-2015-103423

5. Quinlan AR, Hall IM. BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics*. 2010 Mar 15;26(6):841-2. doi: 10.1093/bioinformatics/btq033. Epub 2010 Jan 28. PMID: 20110278; PMCID: PMC2832824.

To further examine the contribution of EGFRwt and EGFRvIII to ligand-dependent signaling, we used GBM14 cells that do not express endogenous EGFR. Expression of EGFRvIII alone results in constitutive signaling and increased invasion (Supplementary Fig. 5B, Supplementary Fig. 1X). Exposure of these cells to EGF has no effect as expected since EGFRvIII does not bind ligand (Supplementary Fig. 5B-C). Similar results were obtained with EGFRwt expression in the absence of ligand. When EGFRwt is expressed alone in GBM14 cells in the absence of ligand there is increased invasion (Fig. 1K-L, Supplementary Fig. 5B-C) and activation of NF- κ B and upregulation of EMP1 (Supplementary Fig. 1T). Ligand-activation of EGFRwt expressed alone in GBM14 cells results in suppression of invasion and upregulation of EGR1 and EMP1 (Fig. 1K-L, Supplementary Fig. 5B-C, Supplementary Fig. 1T))

When we express both EGFRwt and EGFRvIII in GBM14 and express much lower levels of EGFRwt to mimic the conditions in GBM6 and GBM9, we find that addition of EGF still results in suppression of invasion, EGR1 and BIN3 upregulation (Supplementary Fig. 5D-E). In other words, when GBM14 cells expressing a low level of EGFRwt and a higher level of EGFRvIII are exposed to EGF, the ligand-dependent behavior dominates when invasion is examined, just as seen in GBM6 and GBM9. In this experiment we expressed the same level of EGFRvIII but two different levels of EGFRwt (10 fold difference) as shown in Supplementary Fig. 5D-E). Our data suggest that regardless of the level of EGFRwt expressed, when the EGFRwt and EGFRvIII as expressed together in the same tumor, the biological effect of exposure to EGFR ligand on invasion is similar to EGFRwt expression alone

General Comment 3

Third, there is, as the authors themselves have pointed out, evidence from other groups demonstrating a pro-invasion effect for EGFR ligands in some experimental models. The authors claim that this can be explained by EGFR expression levels being low in established GBM cell lines, and provide evidence that through EGFR overexpression in U251 cells EGF turns from being pro-invasive to anti-invasive. However, there are other studies that show this not to be the case. For example, Lund-Johansen et al (PMID: 1523973) showed that EGF induces



invasion/migration in GBM cells with low, medium, and high levels of EGFR expression (as measured by EGF binding, PMID: 2253244). The same study demonstrated that Ab-528, an anti EGFR mAb that blocks EGF binding inhibited invasion/migration. Similarly, Tony Hunter's group showed many years ago (PMID: 11359909) that in cell lines with high level EGFR overexpression, EGF induced cell migration/invasion through inactivation of FAK. Are the authors proposing that this particular mechanism is not functional in GBM cells? Perhaps FAK activation should have been examined. Finally, a study by Stec et al (PMID: 27004406) using the GBM cell line DK-MG, showed that when cells were sorted into high and low-EGFR expressing populations, EGF induced invasion in the former and had no effect on the latter. These studies need to be acknowledged, at the very least. But, more importantly, they raise the possibility that an additional layer of regulation as yet to be identified acts as the toggle that switches pro-invasion into anti-invasion EGFR-dependent functions, and that ligand binding or EGFR expression levels cannot alone explain these differences.

Response:

We appreciate these important comments from the erudite Reviewer and our responses are as follows:

1. In the studies referred to by Reviewer 1, PMID: 2253244, Bigner et al., Cancer Res 1990¹⁰, Lund-Johansen et al., Acta Neuropathologica (PMID: 1523973)¹¹ and the related paper by Lund-Johansen et al., Cancer Res (PMID: 2393868)¹² the authors do indeed report 3 cell lines with different levels of EGFR expression that all respond to EGF with increased invasion that is blocked by Ab-528. It should be noted that all 3 cell lines are established cell lines and likely to have lost the EGFR amplification. None of the 3 lines in the Lund-Johansen paper (D-263 MG, D-247 MG, D-37MG) are available commercially. We also wrote to Professor Morten Lund-Johansen (Morten.Lund-johansen@uib.no) for the cells so that we could test for ourselves, but did not get a response.

However, we were able to find one cell line described in PMID: 1523973, U343MG cells and found that EGF increased invasion in this line. However, when we overexpressed the EGFR in U343MG cells, the response to EGFR switched to suppression of invasion (Supplementary Fig. 2H-I). We also tested two additional non-EGFR amplified Mayo PDX lines (GBM10 and GBM43) that express low levels of EGFR and found that invasion is increased upon exposure to EGF. When EGFR is overexpressed, the response switches to suppression of invasion (Supplementary Fig. 2F-G).

We have acknowledged the relevant study by Lund-Johansen.



2. In the important and interesting study from the Hunter group (Lu et al., 2001)¹³ the authors demonstrated that in high EGFR expressing cells such as A431, MDA-MB-468 and NIH3T3 cells overexpressing EGFR, addition of EGF resulted in increased motility and invasion. Furthermore, they demonstrated that EGF induced dephosphorylation of FAK was required and sufficient for this process. These data seem to conflict with our findings. We have acknowledged this study in our paper.

We examined Fak phosphorylation in multiple EGFR amplified GBM lines and found that EGF consistently *increased* FAK phosphorylation in these lines (Supplementary Fig. 2J). We also used A431 cells and found that FAK was indeed dephosphorylated by EGF (Supplementary Fig. 2J), consistent with the results of the paper published by Lu et al.¹³ Clearly, there are cell type specific differences in whether EGF exposure leads to phosphorylation or dephosphorylation of FAK. However, our findings are consistent with the hypothesis proposed by Lu, et al., that Fak dephosphorylation promotes invasion. In GBM lines, EGF increases FAK phosphorylation and decreases invasion.

3. (PMID: 27004406). In the study by Stec et al,¹⁴ the authors used a DKMG cell line. It is important to note that they used cells with high or low EGFRvIII expression. EGFRvIII does not bind EGFR ligand. *The level of EGFRwt in both the EGFRvIII low and high EGFRvIII cells was the same*¹⁴. Thus, the results showing that EGFR ligand drives invasion in the high EGFRvIII line but not in the low EGFRvIII are difficult to interpret. Perhaps even more importantly, in the Stec et al., invasion experiment, the cells were never directly treated with EGF. In the Stec et al study EGF was used in the bottom chamber as a chemoattractant (page 31912), while we directly treated cells with EGF in all our experiments (EGF added to top chamber). Thus a difference in how the experiment was performed may explain difference in results.

These data indicate that increasing the level of EGFR in glioma cells, followed by exposure to EGFR ligand results in suppression of invasion. This may not be true for tumors derived from other tissues in which ligand-activation of EGFR even in high EGFR expressing tumors may lead to increased invasion, as demonstrated by Lu et. al and we have added this caveat to the paper.

There are also some additional issues, some of which have to do with inconsistencies in the data, or limited interpretation of the data including the following:



Other comment 1. In p19 the authors say, “These data indicate that constitutive EGFR activity drives invasion while ligand-activated EGFR activity suppresses invasion.” What is the evidence that EGFR is constitutively active in these examples (1K-L)? Given that supplementation of EGF in cells with ectopically overexpressed EGFR leads to a level of invasion similar to untreated parental cells, an alternative interpretation is that given sufficient EGFR protein, EGF has no biologically meaningful effects on invasion. In other words, in the presence of sufficient EGF, EGFR expression levels seem irrelevant to invasion capacity (Supplemental 2D, e.g.)

Response: The evidence that constitutive EGFR signaling drives invasion comes from multiple experiments including the data in Fig. 1K-L. The evidence that ligand induced EGFR signaling suppresses invasion is also derived from multiple experiments as outlined below.

Several lines of evidence indicate that EGFR is constitutively active in Fig. 1K-L.

- A. The data shown in Fig. 1K-L is an overexpression experiment performed specifically to test the effect of EGFR expression on invasion in the absence of ligand. EGFR overexpression in the absence of added EGFR ligand results in increased invasion. To exclude an effect of autocrine/paracrine effect of EGFR ligands we showed that cetuximab which inhibits ligand binding to the EGFR, has no effect on the increased invasion in GBM12 cells, further supporting a constitutive (i.e. ligand-independent) effect, (Supplementary Fig. 1P-Q, U-V). The effectiveness of cetuximab in blocking ligand induced tyrosine phosphorylation of the EGFR is shown in Supplementary Fig. 1Q, V. We have done a new experiment to demonstrate that expression of EGFRwt in GBM14 cells, which do not express endogenous EGFR, leads to increased invasion (as shown previously in Fig. 1K-L) and now show that this increased invasion is also not blocked by cetuximab shown in Supplementary Fig. 1R-S).
- B. Furthermore, expression of EGFRwt results in tyrosine phosphorylation of the EGFR (Supplementary Fig. 1S) activation of TAK1 and phosphorylation of p65 and upregulation of EMP1 (Fig. 4K). Importantly, these constitutively generated signals that are triggered by expression of EGFR (for example, phosphorylation p65 and EMP1 upregulation) are unaffected by the addition of EGF (Supplementary Fig. 1T)
- C. In GBM12 expressing endogenous EGFRwt, siRNA to EGFR blocks invasion, suggesting that EGFR drives invasion (Fig. 1C-D). To demonstrate that EGFR signaling driving invasion is constitutive we show that cetuximab (which blocks ligand binding) in GBM12 does not block invasion induced by EGFR overexpression (Supplementary Fig. 1P-Q). This is also true for GBM14 expressing EGFRwt (Supplementary Fig. 1R-S)

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Addition of ligand significantly suppresses invasion.

The Reviewer astutely points out that the level of invasion under resting conditions in the absence of EGFR ligand is similar to EGFR overexpressing cells treated with EGF. “Given that supplementation of EGF in cells with ectopically overexpressed EGFR leads to a level of invasion similar to untreated parental cells, an alternative interpretation is that given sufficient EGFR protein, EGF has no biologically meaningful effects on invasion.” While this is a brilliantly argued point, we would respectfully point out that the key here is the **relative change** in invasion (suppression of invasion) with EGF consistently seen across multiple cell culture and animal experiments. These are outlined below.

The data suggesting that ligand-dependent EGFR signaling suppresses invasion are presented in

- A. Transwell invasion assays (Fig. 1B, 1C, and multiple other figures). Here we show that addition of EGFR ligands results in a suppression of invasion
- B. Scratch wound healing assays (Fig. 1T-U)
- C. Intravital microscopy (Supplementary Fig. 2K-L)
- D. Invasion assay with neurospheres (Supplementary Fig. 1Y-Z).
- E. An extensive set of orthotopic mouse experiments (Fig. 3, Supplementary Fig. 4).
- F. Single cell migration assays (Supplementary Fig. 6A-D, I-L).

To discuss specifically the data in Fig. 1K-L, two PDX lines are used. GBM12 that expresses EGFR wild type, and GBM14 which does not express endogenous EGFR.

GBM12 cells: there is a decrease in invasion with EGF, and this is also the case in GBM12 cells with EGFR overexpression. Thus, addition of ligand to EGFR overexpressing cells also results in a sharp decrease in invasion. The fact that EGF drives down the invasion in EGFR overexpressing cells to the basal level seen in resting cells does not necessarily mean that EGF has no biological effects on invasion since the starting point is quite different. Thus, EGFR overexpressing cells have a much higher level of basal invasion and this is brought down sharply by EGF. The level of invasion in endogenous EGFR expressing GBM12 is lower than EGFR overexpressing cells and this is also significantly suppressed by EGF. Thus, the data in Fig. 1K-L show that EGF suppresses invasion in GBM12 cells with endogenous EGFR expression as well in cells with EGFR overexpression. Thus, we would respectfully point out that regardless of the level of EGFR expression in GBM12 cells, EGF consistently suppresses invasion.

The other PDX line shown in Fig. 1K-L is GBM14. In GBM14 cells there is no endogenous EGFR so there is no suppression of invasion with EGF. When EGFR is ectopically expressed in these cells, invasion is increased. When EGF is added, invasion is suppressed. This is again consistent with our findings in GBM12 cells and indicates that ligand-independent or constitutive



EGFR signaling results in increased invasion, while ligand-dependent EGFR signaling suppresses invasion.

Relative suppression of invasion

The Reviewer comments that “In other words, in the presence of sufficient EGF, EGFR expression levels seem irrelevant to invasion capacity (Supplemental 2D, e.g.)”

In Supplementary Fig. 2D (now Supplementary Fig. 2E) we show that in the presence of low EGFR expression, EGF increases invasion. When EGFR is overexpressed there is a substantial increase in basal invasion. When EGF is added invasion is suppressed. Thus, two clearly distinct responses are seen. EGF increases invasion in low EGFR expressers and suppresses invasion in high EGFR expressers. Again, the fact that EGF drives down the invasion in EGFR overexpressing cells to the EGF stimulated level seen in low EGFR cells does not necessarily mean that in the presence of sufficient EGF, EGFR expression level has no biological relevance to invasion since the starting point is quite different. **We propose that it is the relative change in invasion that is the key readout.** The experimental data clearly demonstrate that EGFR expression is a key determinant of the invasion response so that in low EGFR expressers, EGF increases invasion, while in high EGFR expressers EGF suppresses invasion.

Thus, if we have two GBMs

GBMa has high EGFR expression and is exposed to high ligand. In these tumors, basal invasion level is high. EGF will suppress invasion and reduce invasion from the basal high level of EGFR expression. This will be a favorable dynamic change in this tumor. This is what we propose happens in EGFR amplified GBMs with high EGFR ligand.

GBMb has low EGFR expression and is exposed to high ligand: In these tumors the basal invasion level is low. The addition of EGF increases invasion and results in an unfavorable dynamic change in this tumor. This is likely the situation in non EGFR amplified GBMs with high ligand.

This conceptual framework holds even if the eventual level of invasion in GBMa or GBMb is similar. We propose that it is the dynamic change in invasion that is the key determinant of tumor behavior and such invasive behavior seems causally linked to EGF receptor and ligand levels in GBM as is consistently demonstrated in multiple models in our study. We acknowledge that we are dealing with a complex biological system and that there are likely multiple additional factors (one example is Fak) that influence the invasive behavior of GBMs. We also acknowledge that this model may not hold for certain other types of cancer. Our data do suggest that EGFR and EGFR ligands are an important component of the regulation of invasion in GBMs.



Other Comment 2. In Figure 1N, EGFR is not at all overexpressed in GBM9 cells transduced with the WT EGFR construct, but a significant increase in cell invasion is still observed. In fact, there may even be a decrease in EGFR in the transduced line.

Response: We have relabeled the Figure more clearly. GBM9 has substantial basal EGFRvIII expression. EGFRvIII is truncated and migrates lower than EGFRwt on electrophoresis. We have labeled EGFRwt and EGFRvIII in the Figure. EGFRwt (the upper band) is clearly increased in GBM9 cells transfected with EGFRwt.

Other Comment 3. The authors argue that “ligand-activated EGFR activity suppresses invasion.” However, the cetuximab experiments show that blocking ligand binding does not affect invasion. This would assume that there is no ligand-bound EGFR under the steady-state conditions of growth under which the invasion assays are carried out. This is simply not true. In fact, the western blot shown in Supplementary Figure 1N shows that in the absence of added EGF, EGFR phosphorylation goes down with cetuximab treatment (lanes 1 vs 3). Also, the authors failed to show that pre-treating cells with cetuximab prevented EGF-induced suppression of cell invasion.

Response: This is an important point. We undertook an experiment to examine whether pretreatment with cetuximab blocks EGF induced suppression of cell invasion. As expected, cetuximab blocks the EGF mediated suppression of invasion (Supplementary Fig. 1U-V). We also undertook an experiment in GBM14 cells that do not express endogenous EGFR. We transfected GBM14 cells with EGFR and found the expected increase in invasion. This increased invasion is not blocked by cetuximab suggesting that it is mediated by ligand-independent EGFR signaling. When we add EGF, invasion is suppressed. The use of cetuximab blocks the EGF mediated suppression of invasion in GBM14 cells expressing EGFR (Supplementary Fig. 1R-S). These observations are consistent with our finding that cetuximab does not influence invasion in GBM12 cells.

The small amount of basal EGFR phosphorylation present in GBM12 does appear to go down with cetuximab (Supplementary Fig. 1N—now Supplementary Fig. 1Q), suggesting the presence of small amounts of ligand in the milieu. However, the fact that cetuximab does not influence invasiveness of these cells suggests that invasion in GBM cells is primarily driven by constitutive EGFR signaling (blocked by erlotinib and siRNA knockdown of EGFR (Fig. 1C-F, Fig. 1I—but not cetuximab, Supplementary Fig. 1P). This was confirmed in a second PDX line. We expressed EGFRwt in GBM14. EGFRwt expression resulted in increased invasiveness that was not blocked by pretreatment with cetuximab. When EGF was added invasion was



suppressed and this was blocked by cetuximab (Supplementary Fig. 1R-S). These data support our model that constitutive EGFR signaling drives invasion (no effect of cetuximab) while ligand-activated EGFR signaling suppresses invasion (blocked by cetuximab). We further infer from these data that the level of ligand-activated EGFR in our experimental system under basal conditions may be too low to influence invasion.

Other Comment 4. Although they show that GBM14 cells ectopically expressing EGFRvIII have enhanced invasion, they do not show whether EGF is ineffective at blocking invasion in these cells.

Response: This experiment has been done and the data are shown in Supplementary Fig. 1W-X. EGF has no effect on invasion when the non-ligand binding mutant EGFRvIII alone is expressed.

Other Comment 5. To make any claims about U251 EGFR levels being low, they need to run them alongside their PDX-derived and neurosphere cells for comparison. GSC11 cells, for example, have “low” EGFR levels but invasion is also inhibited by EGF in these cells.

Response: Supplementary Fig. 2D shows the side by side comparison of U251 and GSC11 EGFR levels showing that the EGFR levels in GSC11 are higher than U251 cells. We acknowledge that there are likely additional factors that regulate invasiveness of cells and these may influence the differential effects of EGFR signaling on invasion. We would point out that in any cell line that we have tested, an experimental increase in EGFR level promotes invasion and when EGF is added, invasion is suppressed (Fig. 1K-L). Also, in low EGFR expressing lines, EGFR ligand promotes invasion while EGFR overexpression changes the response to ligand from increased invasion to suppression of invasion (Supplementary Fig. 2A-I).

Other Comment 6. Others have previously shown that BIN3 depletion increases invasion (PMID: 18339847). In fact, Fig 1R could be read as “in the presence of EGF, loss of BIN3 increases cell invasion.”

Response: PMID: 18339847 by Ramalingam et al., reported that BIN3 deletion causes cataracts and increased susceptibility to lymphoma during aging and demonstrated that BIN3 loss promotes motility in transformed cells¹⁵. Thus, a putative tumor suppressor role of BIN3 and other BAR domain proteins such as BIN1¹⁶ has previously been noted and provides strong support to our model of a key role for BIN3 in EGFR induced suppression of invasion. BIN3 overexpression suppresses invasion (Fig. 2A-B, Fig. Supplementary Fig. 4N-R). This effect does not require administration of EGF. We show that ligand-activated EGFR signaling



suppresses invasion and upregulates BIN3. If BIN3 is silenced then ligand-induced EGFR activation is no longer able to suppress invasion (Fig. 1R-S, Fig. 3L-Q). We infer from these findings that ligand-mediated EGFR activation suppresses invasion via an upregulation of BIN3. We acknowledge that an alternative reading of Fig. 1R as suggested by the Reviewer could be “in the presence of EGF, loss of BIN3 increases cell invasion.” This alternative reading is consistent with our finding that BIN3 is tumor suppressive and does not contradict our model or hypothesis.

Other Comment 7. It is unclear why the addition of EGF does not inhibit invasion in cells that overexpress DOCK7 (Supplementary 2R) despite EGF still being able to suppress RhoA activation (Supplementary 2S).

Response: The DOCK7 overexpression Western blot was repeated and all the lanes for a particular line were run on the same gel. DOCK7 overexpression results in increased RhoA and Cdc42 GTPase activity. EGF is not able to suppress RhoA or Cdc42 GTPase activation in DOCK7 overexpressing cells, while it continues to do so in control cells Supplementary Fig. 2X (the previous Supplementary Fig. 2R-S). This is consistent with the invasion assay showing that DOCK7 overexpression increases invasion and results in a loss of EGF mediated suppression of invasion as shown in Supplementary Fig. 2W.

Other Comment 8. In figure 2I, the increase in BIN3 levels in DOCK7 immunoprecipitates from EGF-treated cells tracks with the increase in BIN3 levels seen in whole cell lysates. This might suggest that the association between BIN3 and DOCK7 is constitutive and therefore primarily regulated by protein expression levels. However, in figure 2J, the levels of DOCK7 associated with BIN3 immunoprecipitates seem to increase following EGF treatment despite unchanged levels of DOCK7 in whole cell lysates, and similar levels of BIN3 in all immunoprecipitates. This would instead indicate that the BIN3/DOCK7 interaction might be regulated not only by BIN3 levels, but also by other EGF-dependent events. The authors need to explain this.

Response: We considered the possibility that administration of EGF results in a post-translational modification that facilitates a BIN3-DOCK7 association. Since EGFR is a tyrosine kinase, we considered the possibility that either BIN3 or DOCK7 could become tyrosine phosphorylated by ligand-activated EGFR. Indeed, **we found that DOCK7 becomes tyrosine phosphorylated upon EGF treatment as shown in Fig 2Q-R.** A previous study has reported that DOCK7 becomes tyrosine phosphorylated on Y1118¹⁷. We also found that EGF stimulation



results in tyrosine phosphorylation of DOCK7 using the phospho Y1118 DOCK7 antibody that is commercially available.

Mutation of this (Y1118) residue results in a loss of DOCK7 phosphorylation in response to EGF and a loss of DOCK7-BIN3 association (Fig. 2S-T).

We found that BIN3 does not become tyrosine phosphorylated in response to EGF (Fig. 2U)

Thus, new experimental data support what is suggested by Reviewer 1 and provide a key mechanistic insight into the interaction of BIN3 and DOCK7, suggesting that EGF induced tyrosine phosphorylation of DOCK7 is required for its association with BIN3.

Other Comment 9. Figure 4H shows no interaction between EGFR and TAB1 in parental GBM12 cells despite the decrease in invasion seen with EGFR siRNA in these cells (Figures 1C and 1G), and the observed interaction when assessed by M/S (Figure 4G). How do you reconcile the fact that EGFR siRNA inhibits invasion, but TAB1 siRNA does not?

Response: We repeated the experiment to examine the interaction between TAB1 EGFR by coimmunoprecipitation in multiple lines shown in a new Fig. 4H. We are able to detect some basal interaction between TAB1 and EGFR in GBM12 and GBM9. When EGFR is overexpressed the TAB1-EGFR association is increased. Addition of EGF results in a dissociation of EGFR and TAB1 (Fig. 4H) consistent with the previously reported mass spec studies (Fig. 4G).

As for why EGFR siRNA inhibits invasion but TAB1 siRNA does not, we suggest that TAB1 activity is required for EGFR mediated invasion but not sufficient by itself to drive invasion. We are dealing with a complex biological system. We have identified a core pathway involving TAB1 that drives invasion, here but there are likely additional relevant EGFR driven signals that drive invasion.

Other Comment 10. Given that EGFR amplification is a feature of the classical transcriptional subtype (which has distinct biology), is it not possible that the prognostic value of EGFR ligands is linked to the transcriptional subtype rather than EGFR levels? Can this be evaluated?

Response:

We examined the response to EGFR ligand in the transcriptional subtype. 55 out of the 149 GBMs in TCGA are classified as classical. We do not detect a prognostic value of EGFR ligand



in the transcription subtype (Fig. 7I). This is likely because not all patients in the classical transcriptional subtype are EGFR amplified. Using the same criteria used for all GBMs (EGFR copies >4, Fig. 7B), we found that 15 out of the 55 patients have <4 EGFR copies. The data in Fig. 7C clearly show that high EGFR+high EGFR ligand confers a better prognosis, suggesting that it is the EGFR level --and not other features of the transcriptional subtype --that is the primary determinant of the prognostic response to EGFR ligands.

Other Comment 11. In p33 the authors state, “Although constitutively active mutants are expressed in GBM, the most common EGFR type expressed in GBM is EGFRwt. While expression of EGFRwt is associated with a low level of EGFR phosphorylation, addition of EGFR ligand results in a much greater phosphorylation of the EGFR (Supplementary Fig. 5I), suggesting that overall EGFR phosphorylation in GBM is likely to largely reflect ligand-activated EGFRwt.” While this is true, the frequency of EGFR mutation (including high level focal amplification which leads to ligand-independent activation) is high enough that it could drive the enrichment.

Response: We acknowledge this possibility and have modified the text accordingly.

Minor points:

Minor Comment 1. GBM14 should have been included as a negative control in Figure 1O (i.e. in response to EGF).

Response: The experiment was done and the data are shown in Fig. 1O.

Minor Comment 2. Figure 3Q is missing statistics

Response: Fig. 3Q is now Fig. 3O and includes statistics

Minor Comment 3. Figure 5K, increase in pEGFR following STAT3 KD is marginal, despite significant increase in BIN34

Response: A repeat of the pEGFR Western blot shows a more clear result with significant increase in pEGFRwt following STAT3 knockdown that is blocked by cetuximab.

Minor Comment 4. In Figure 6R, should you not expect that knockdown of HB-EGF increase invasion?



This is an excellent point. Our data indicate that overexpression of EGFR ligand suppresses invasion but decrease in HB-EGF does not decrease invasion (Fig. 6R). We propose that ligand-activated EGFR suppresses invasion via an upregulation of BIN3. BIN3 exerts its anti-invasion effect by inhibiting DOCK7 by binding to it. DOCK7 becomes tyrosine phosphorylated in response to EGF and makes it responsive to binding to BIN3 and to inhibition by BIN3. Under basal conditions in the absence of EGFR ligand, DOCK7 is constitutively active and activates Rho GTPases that drive invasion. Under basal conditions the low levels of BIN3 available does not significantly bind to unphosphorylated DOCK7 (Fig. 2I-K). Thus further decreasing HB-EGF cannot further decrease a non-existent or a very low level BIN3-DOCK7 association. These data provide an explanation for why silencing HBEGF cells does not lead to increased invasion.

On the other hand, increased EGFR ligand leads to increased BIN3 levels, tyrosine phosphorylation of DOCK7 and an increased association of BIN3 with DOCK7 which inhibits DOCK7 activity leading to suppressed invasion. Thus, in our experimental system, this is a gain of function property of HB-EGF (EGFR ligand).

Minor Comment 5. Although the effects of erlotinib were measured at both 24 and 48hrs, the effects of EGF stimulation were only assessed at 24hrs throughout the manuscript.

Response: The experiment was done to include a 48h timepoint and the data are shown in Supplementary Fig. 1D.

Minor Comment 6. Authors state, “A time course of the erlotinib effect and its impact on signaling is shown in Supplementary Fig. 1J-L.” Supplementary Figure 1 does not show any time-course signalling data following erlotinib treatment. Also, there are only two time points which barely qualifies as a time course.

Response:

Additional time points were done and invasion assay and effects on pEGFR and BIN3 are shown in Supplementary Fig. 1K-L.

Final Comment: For all these reasons, I cannot recommend publication of this manuscript in Nature Cell Biology unless all of these points have been satisfactorily addressed.



Response: We appreciate the thorough and incisive critique, and have tried to address all of the concerns raised by Reviewer 1.

Reviewer #2:

Remarks to the Author:

General Comment: This is a complex and dense paper by a thought-leader in the field, that makes a number of new and important insights into EGFR signaling in glioblastoma, examining the apparent paradoxical effects of ligand binding in blocking invasion while promoting proliferation. While one would anticipate that ligand binding would thus lead to a contest, balancing increased proliferation against decreased invasion, experiments proposed show that tumors are both smaller and less invasive (despite showing higher levels of proliferation) a paradox that is not explained.

While experiments in this paper are done to a high standard, and are generally convincing, the stories here are both dense and complicated. The authors might consider stripping the invasion/proliferation story to make this a simpler tale (Figs 1-3 and 7) and then writing a second paper that incorporates details and translational importance from Figs 4-6.

Response: We appreciate the thoughtful critique and the positive comments. This is indeed a complex system that is not fully understood. However, to our knowledge, this is perhaps the first experimental system in which you can suppress invasion and increase proliferation simultaneously. And the results are indeed quite surprising.

We acknowledge that the paper has a lot of data and certainly if we were writing this paper de novo, we could consider dividing the data into two papers. However, with the editorial recommendations and responding to critiques from the other reviewers, it would be very difficult to omit large parts of the data from the study at this point. So we have retained the data in one paper and have tried to organize the data in a coherent narrative.

Specific Comment 1. Authors state that, "the level of EGFR expression determines whether the outcome of ligand-dependent EGFR signaling is increased or decreased invasion". Can this result (that ligand activation activates invasion in cells with low EGFR) be validated in additional EGFR-low GBM lines? In this regard, why don't cells in Figs 1C-1F show increased invasion in response to siEGFR + EGF?

Response: We now show that EGF promotes invasion in two additional Mayo PDXs, GBM10 and GBM43 that express a low level of EGFR. If we increase the EGFR levels in the same lines,

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now EGF suppresses invasion. The data are shown in Supplementary Fig. 2F-G. Additionally, we have similar results in an established GBM line U343MG (Supplementary Fig. 2H-I).

In Fig. 1C-F we show the results of silencing EGFR on invasion. The Reviewer correctly points out that in EGFR silenced cells a small amount of EGFR is still expressed, and why do these cells not respond to EGF with increased invasion?

This is because the residual EGFR is EGFRvIII that does not bind EGF. In Figure 1C siRNA knockdown of EGFR was done in GBM12 and GBM6. GBM12 expresses EGFR wild type (EGFRwt) only. GBM6 expresses a low level of EGFRwt and a high level of EGFRvIII (shown in a revised Fig. 1D). EGFR siRNA knockdown suppresses EGFRwt completely in GBM12 while in GBM6 there is a low level of remaining EGFRvIII. In a revised Fig. 1F we show EGFR knockdown in neurospheres GBM9 and GSC11. GBM9 expresses a high level of EGFRvIII and a low level of EGFRwt. EGFR siRNA knockdown completely suppresses EGFRwt in GSC11 while in GBM6 and GBM9 there is a low level of remaining EGFRvIII. EGFRvIII does not bind EGFR ligand and thus addition of EGF would not influence invasion in these cells. We further examined this by subjecting GBM12 and GBM9 to EGFR siRNA and examining if there is functional EGFRwt in these cells. We did pEGFR and pERK blots in response to EGF in these knockdown cell lines and found no activation of EGFR or ERK in these cells (Supplementary Fig. 1I). These new data clearly indicate the ligand-activated EGFR pathway is completely silenced/blocked in these cells and the very low level of EGFR remaining is insufficient to generate signaling. We also show data for U251 and GS622 as positive controls (Supplementary Fig. 1J).

Specific Comment 2. In the setting of erlotinib treatment over time (Fig S1L), does erlotinib lead to reduced expression of BIN3, and do cells that recover from erlotinib induced suppression of invasiveness show re-expression of BIN3?

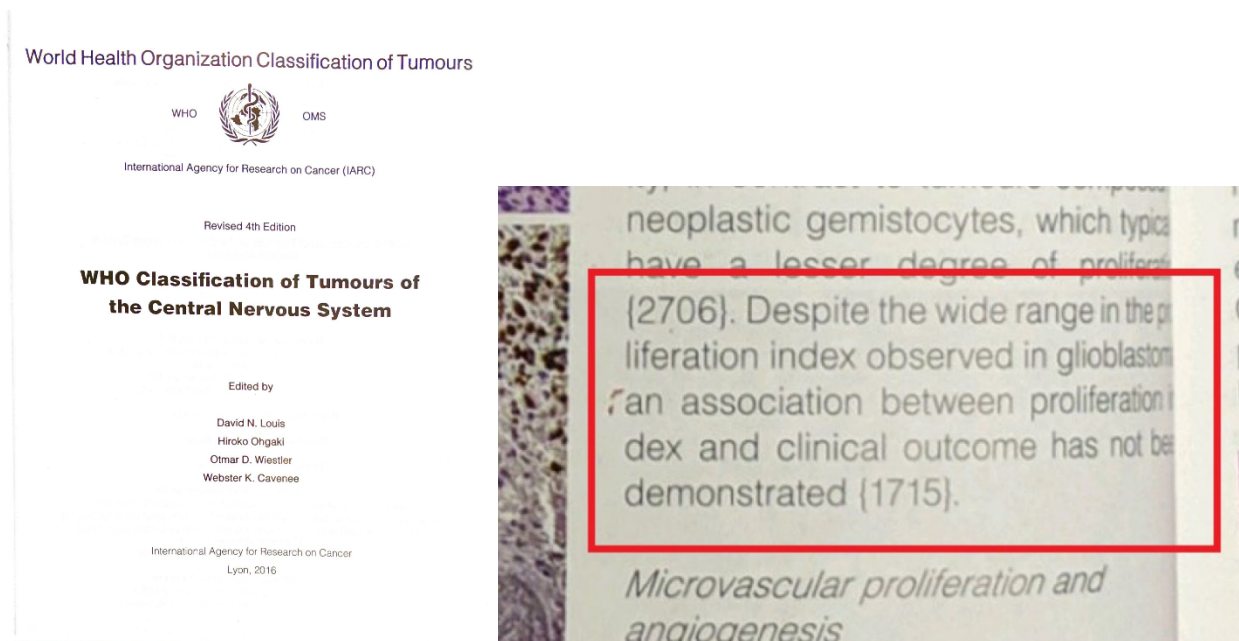
Response: We found that erlotinib does not alter levels of BIN3 (Fig. 1J, Supplementary Fig. 1L). In combination with the other findings of our study, this observation suggests that constitutive EGFR signaling does not suppress BIN3 levels. As shown in Fig. 4 a TAB1-TAK1-Nf- κ B-EMP1 pathway drives invasion in response to constitutive EGFR signaling. Ligand-activated EGFR on the other hand upregulates BIN3 that then binds to DOCK7 to inhibit the activity of DOCK7(Fig. 2).



Specific Comment 3. In Fig 4, how do authors reconcile apparently conflicting results of higher proliferation resulting in smaller tumors?

Response: Unrestrained proliferation is a hallmark of cancer and is certainly required for tumor growth. Our data suggest that proliferation alone is not sufficient for tumor expansion in GBM and indicate a requirement for invasion for tumor expansion in GBM. Thus, ligand-activated EGFR signaling in EGFR amplified GBMs or high EGFR expressing cells both increases proliferation and suppresses invasion with the net result being inhibition of tumor expansion and smaller tumors. *We consider this a novel and important finding of this study.* This is also quite consistent with TCGA analysis showing that high EGFR ligand in EGFR amplified GBMs confers a better prognosis compared to low ligand (Fig. 7D). The experimental data are shown in Figure 3 and Supplemental Figure 4.

It is important to note that evidence demonstrating an independent prognostic role for Ki-67 or MIB1 (a marker of proliferation in GBM) has been elusive in GBM, at least in some studies¹⁸. We refer Reviewer 2 to the WHO classification of brain tumors (2016), the authoritative reference for neuropathologists. This text also comments on the lack of a correlation between Ki-67 and MIB-1 and cites the same reference as we have¹⁸. We have inserted the paragraph of the relevant page here.



We propose that this is a new biological insight from our study—that invasion is required for tumor expansion and that proliferation alone is not sufficient.

Specific Comment 4. IP in Fig 4H shows very poorly focused EGFR band, can this be repeated?

Response: The experiment has been repeated and a better quality image is presented.

Specific Comment 5. Early figures in the manuscript suggest that Dock7 is the major driver of invasion, and later figures demonstrate the importance of EMP1. Are these parallel or intersecting pathways that promote invasion?

Response: This is an important point. They are intersecting pathways in that both are involved in downstream activation of Rho GTPases, key mediators of invasion. But they are triggered by different upstream mechanisms. EMP1 is upregulated by constitutive EGFR signaling and not ligand-activated EGFR signaling (Fig. 4A, Fig. 4C). DOCK7 is constitutively active and suppressed by ligand-activated EGFR signaling. Both pathways are involved in activation of Rho GTPases as shown in Fig. 4F, Fig. 2M, and Supplementary Fig. 3I.



Reviewer #3

Remarks to the Author:

General Comment: In the paper “EGFR ligand shifts the role of EGFR from oncogene to tumor suppressor in EGFR amplified glioblastoma” Guo, et al. claimed that in EGFR amplified GBMs EGF inhibited the invasion of GBMs. The results were of interest and the conclusion was pretty challenging, however, there were some major issues which need to be very clearly addressed, to support the main conclusion.

Response: We appreciate reviewer comments that our results are of interest and the conclusion is challenging. We have tried to address all of the issues raised by the Reviewer.

Major :

Comment 1. The most critical concern of mine is that how can we define constitutive EGFR signaling? It was not clearly described in the paper. Was it decided by the level of EGFR only? If so, Knockdown of EGFR make GBMs from constitutive to ligand signaling, and overexpress of EGFR shifts GBMs from ligand inducing signaling to constitutive signaling. In EGFR knockdown cells, treatment of EGF will enhance the invasion just like GBM622 while your results indicated that the invasion percentage rarely changed in Figure 1E. if it still harbors the character of constitutive signaling. It's obvious not acceptable. If it was decided by the reaction to EGF, it was too subjective to measure by objective experiments.

Response:

We apologize for the failure to define constitutive EGFR signaling clearly. **Constitutive EGFR signaling is ligand-independent EGFR signaling.** There are seven known EGFR ligands or growth factors (EGF, HB-EGF, TGF α etc) expressed in GBM (Fig. 7A) that bind to EGFR and activate it. However, EGFR can also be activated and generate downstream signals in the absence of EGFR ligand. This is termed constitutive signaling. Thus, **constitutive signaling** is defined as signaling triggered by EGFRwt or EGFRvIII expression leading to spontaneous dimerization and downstream **signaling in the absence of EGFR ligand**^{19, 20}. There is substantial evidence from previous studies that both constitutively active EGFR mutants as well as EGFR wild type (EGFRwt) can signal constitutively in the absence of ligand¹⁹⁻²². Thus, constitutive EGFR signaling is ligand-independent EGFR signaling. **The key difference is presence or absence of EGFR ligand, not the EGFR level.**

Our data indicate that constitutive EGFR signaling drives invasion in EGFR amplified GBMs. When ligand is added, invasion is suppressed.



Knockdown of EGFR in GBM PDXs results in a loss of invasion (Fig. 1C-D). This results from a loss of constitutive or ligand-independent EGFR signaling. EGFR signals ligand-independently or constitutively to drive invasion since cetuximab, which inhibits ligand binding to the EGFR fails to inhibit constitutive EGFR signaling mediated invasion – both for endogenous EGFR and experimentally overexpressed EGFR (Supplementary Fig. 1P-V). Also expression of the EGFRvIII mutant --which is missing the ligand binding domain of EGFR and thus cannot bind ligand and signals constitutively -- results in increased invasion (Supplementary Fig. 1W-X).

The other important finding is that when EGFR level is low, EGFR ligand promotes invasion while if the EGFR level is high, EGFR ligand suppresses invasion.

In the data shown in Fig. 1C-F we have silenced EGFR in various lines, leading to decreased invasion. The reviewer correctly points out that there is some remaining EGFR in the silenced cells and they should behave (like the low EGFR expressing GS622) to EGF with increased invasion. However, our data indicate that there is no increase in invasion. This is because the remaining EGFR is GBM6 and GBM9 is EGFRvIII, the EGFR mutant that does not bind ligand.

In Figure 1C siRNA knockdown of EGFR was done in GBM12 and GBM6. GBM12 expresses EGFR wild type only. GBM6 expresses a low level of EGFRwt and a high level of EGFRvIII (shown in a revised Fig. 1D). EGFR siRNA knockdown suppresses EGFRwt completely in GBM12 while in GBM6 there is a low level of remaining EGFRvIII. In Fig. 1F we show EGFR knockdown in neurospheres GBM9 and GSC11. GBM9 expresses a high level of EGFRvIII and a low level of EGFRwt (shown in a revised Fig. 1F). EGFR siRNA knockdown suppresses EGFRwt completely in GSC11 while in GBM6 and GBM9 there is a low level of remaining EGFRvIII. EGFRvIII does not bind EGFR ligand²³ and thus addition of EGF would not influence invasion in these cells. We further examined this by subjecting GBM12 and GBM9 to EGFR siRNA and examining if there is functional EGFRwt in these cells. We did pEGFR and pERK blots in response to EGF in these knockdown cell lines and found no activation of EGFR or ERK in these cells (Supplementary Fig. 1I). These new data clearly indicate the ligand-activated EGFR pathway is completely silenced/blocked in these cells and the very low level of EGFR remaining is EGFRvIII which cannot bind EGFR ligand and thus cannot generate ligand-dependent signaling. We also show data for U251 and GS622 as positive controls (Supplementary Fig. 1J).

Comment 2. How to evaluate different invasion status regarding the level of p-EGFR? As you



mentioned in the paper, constitute or ligand induced EGFR signaling both promotes the phosphorylation of EGFR but resulted in different results.

Response: The Reviewer raises an important point.

EGFR phosphorylation: We propose that the level of tyrosine phosphorylation of EGFRwt plays a key role in driving the distinct downstream cascades and biological outcomes triggered by constitutive vs. ligand induced signaling. Although the EGFR is phosphorylated on the same phosphotyrosine sites in both constitutive and ligand-activated EGFR signaling, there is a big difference in the level of tyrosine phosphorylation of the EGFR induced by constitutive/ligand-independent or ligand-dependent EGFR activation. Our data show that constitutive or ligand-independent EGFR signaling results in a low level of EGFR phosphorylation at Y1068, Y1173, and Y845, while addition of ligand results in a much more robust phosphorylation of the EGFR on the same residues (Supplementary Fig. 5N, Supplementary Fig. 1T).

GBM14 does not express endogenous EGFR. To experimentally test this hypothesis and conceptual framework we introduced EGFRwt into GBM14 followed by analysis of EGFR phosphorylation (3 different residues), When EGFR is introduced into GBM14 cells, there is a low level of tyrosine phosphorylation of Y1068, Y1173 and Y845 that increases substantially with EGF (Supplementary Fig. 1T). This is similar to what is seen in in GBM12 and GBM9 (Supplementary Fig. 5N). Thus, constitutive EGFR signaling results in low level EGFR phosphorylation while ligand-dependent EGFR signaling results in a high level of EGFR phosphorylation. How this difference in the level of EGFR phosphorylation translates into distinct downstream mechanisms is outlined below.

Comment 3: The mechanism remains largely unknown, the main theme of this paper should focus on the different status of EGFR signaling in constitute signaling or ligand induced signaling such as the different phosphorylation site or spatial change, and the downstream cascade. If EGFR signaling status was not changed, why different downstream cascade happened? If the mechanism was still unclear and we thought that EGF affect other unknown receptors and EGFR signaling was not a dominate.

Response:

We identified two distinct mechanisms and downstream signaling cascades that mediate the different biological effects of constitutive vs. ligand-activated EGFR signaling.

Brief Summary:



Constitutive EGFR signaling: We propose that a low level of EGFR phosphorylation detected in constitutive EGFR signaling is conducive to TAB1 binding. This results in the association of TAB1 with the EGFR and leads to activation of a TAB1-TAK1-NF-kappaB-EMP1 pathway that promotes invasion (Fig. 4).

Ligand-dependent EGFR signaling results in a dissociation of TAB1 from the EGFR (Fig. 4H), robust tyrosine phosphorylation of the EGFR followed by induction of EGR1, upregulation of BIN3 which associates with DOCK7 and suppresses DOCK7 activity resulting in decreased Rho GTPase activity and suppression of invasion (Fig. 1 and 2).

Detailed response:

EGFR phosphorylation: We propose that the level of tyrosine phosphorylation of EGFRwt may play a key role in the distinct downstream cascades and biological outcomes triggered by constitutive vs. ligand induced signaling. Although the EGFR is phosphorylated on the same phosphotyrosine sites in ligand-independent/ constitutive vs. ligand activated EGFR, there is a big difference in the level of tyrosine phosphorylation of the EGFR induced by ligand-independent or ligand-dependent EGFR activation (Supplementary Fig, 5N). The data in Supplementary Fig. 5N show that constitutive or ligand-independent EGFR signaling results in a low level of EGFR phosphorylation at Y1068, Y1173, and Y845, while the addition of ligand results in a much more robust phosphorylation of the EGFR on the same residues.

Downstream cascades: discussed in detail below. We propose that a low level of EGFR phosphorylation induced constitutive EGFR signaling is conducive to TAB1 binding, leading to activation of a TAB1-TAK1-Nf-κB –EMP1 pathway that drives invasion.

On the other hand, a high level of EGFRwt phosphorylation results in dissociation of TAB1 from the EGFR (Fig. 4H) and triggers an alternative pathway driven by EGR1-BIN3-DOCK7 that suppresses of invasion (Fig. 1-2).

New experiment:

GBM14 does not express endogenous EGFR. To experimentally test this hypothesis and conceptual framework we introduced EGFRwt into GBM14 followed by analysis of EGFR phosphorylation (3 different residues), pp65 activation, and assays of invasion. We also examined BIN3 and EGR1 levels which are not upregulated. It should be noted that comparisons of constitutive and ligand-activated signaling can only be done for EGFRwt, since the EGFRvIII mutant is missing the ligand binding domain and does not bind ligand²⁴.

These results indicate the following:



When EGFR is introduced into GBM14 cells, there is a low level of tyrosine phosphorylation of Y1068, Y1173 and Y845 and NF- κ B as measured by phosphorylation of pp65 is activated, EMP1 is upregulated (Supplementary Fig. 1T) and invasion is increased (Fig. 1K-L).

When EGFR is introduced into GBM14 cells and cells are exposed to EGF, there is a large increase in phosphorylation of Y1068, Y1173 and Y845 (Supplementary Fig. 1T). EGR1 and BIN3 are upregulated and invasion is suppressed.

As noted above, there is a big difference in the tyrosine phosphorylation of the EGFR induced by ligand-independent or ligand-dependent EGFR activation. In this study we have identified distinct mechanisms that mediate the differential effects of ligand-independent and ligand-dependent EGFR signaling. **Thus we have provided substantial new mechanistic insights as detailed below**

1. Constitutive or ligand-independent EGFR signaling EGFR mediates invasion. We used an unbiased mass spectrometry approach and identified TAB1 as a protein that binds to EGFR only in the absence of EGF. TAB1 is linked to the activation of a TAK1-NF-kappaB-EMP1 pathway. With a careful loss of function experiments for each component we identified this pathway as being required for EGFR mediated invasion. The data are shown in Fig. 4. The link to EGFR phosphorylation is discussed above in response to Reviewer 3 Comment 2. Thus the low level of EGFR phosphorylation – but not phosphorylation of a specific residue--correlates with activation of the TAB1-TAK1-NF- κ B-EMP1 pathway.
2. How ligand-dependent EGFR signaling suppresses invasion: Ligand-dependent EGFR signaling upregulates the cytoskeletal N-BAR domain containing protein BIN3 (Fig. 1P). BIN3 is not upregulated by constitutive or ligand-independent EGFR activity (Supplementary Fig 2C, G, Supplementary Fig. 1T). BIN3 associates with DOCK7 and inhibits the activity of a constitutively active DOCK7- RhoGTPase pathway resulting in suppressed invasion (Fig. 2H-P). BIN3 is required for this EGFR mediated inhibition of invasion, in vitro and in vivo (Fig. 1R-S, Supplementary Fig. 2R-S, Fig. 3N-Q). Furthermore, BIN3 overexpression also suppresses invasion in vitro (Fig. 2A-B) and in vivo (Supplementary Fig. 4 P-R). We identified EGR1 as a transcription factor activated by ligand mediated activation of the EGFR that plays a key role in ligand-activated EGFR mediated upregulation of BIN3 (Fig. 2C-G).

The effects of EGFR on invasion are specifically related to EGFR and not some other receptor²⁵based on the following observations

1. siRNA to EGFR downregulates invasion



2. Overexpression of EGFR increases invasion.
3. siRNA knockdown of EGFR abolishes the invasion suppressive effect of EGF.

It should be noted that EGFRvIII is known to be constitutively active and does not bind EGFR ligand. GBM9 cells express high level of EGFRvIII and the EGF induced responses in these cells result from activation of co-expressed EGFRwt.

Comment 4. The microarray you used in ref 2 was not suitable here. You compare EGFR-low cells with EGFR-low + EGFR cells. This was not correct because the genetic background of EGFR-low cells and EGFR amplified cells were different. In this paper, you should collect GBMs (GBM6 GBM12) treated with or without EGF and subjected to RNA sequence. Thus, the selection of BIN3 was unconvincing. Moreover, the microarray data was applied in U251MG, which was not acceptable in this paper, GSC should be applied instead. Your description “We found that 93 genes were upregulated by EGFR overexpression in glioma cells in the absence of exogenous EGF while 66 genes were upregulated only when EGF was added” didn’t match with the real sequence strategy as well.

Response: The previous experiment in our published paper (ref 2)²⁵ was done in an established isogenic glioblastoma cell line U251MG cells. EGFR was inducibly expressed in this cell line using a tetracycline inducible system and the transcriptional response to EGFR expression with or without EGF was examined. The genetic background of U251 cells was identical in all conditions for this experiment with the exception of increased EGFR expression.

BIN3 was identified on the basis of this study as a gene that is exclusively upregulated by ligand-dependent and not by ligand-independent or constitutive EGFR signaling.

In the current study, the EGF mediated upregulation of BIN3 was rigorously validated by qPCR and Western blot in multiple PDX and neurosphere lines (Fig. 1P-Q). Furthermore, we demonstrate in a large amount of convincing experimental data that BIN3 overexpression suppresses invasion in vitro and in mouse models, and that BIN3 is required for EGFR mediated suppression of invasion in vitro and in vivo.

Thus, although the original observation of BIN3 upregulation by EGF was made in an established glioblastoma cell line:

1. The experiment is scientifically rigorous since it was done in an isogenic U251 cell line by experimentally expressing EGFR.
2. The BIN3 upregulation is now fully validated by extensive data in contemporary glioma models using multiple PDX including GSCs.



.The information about 93 genes and 66 genes being altered in the previous study has been removed since it is not relevant here.

Comment 5. There were some contradictions in Figure 3Q-S. BIN3 was claimed as a tumor suppressor. Why knocking down of BIN3 had no influence on invasion percentage and the overall survival of mouse?

Response: This is an excellent point. Our data indicate that overexpression of BIN3 suppresses invasion and improves survival but decrease in BIN3 does not decrease invasion or worsen survival. We propose that ligand-activated EGFR suppresses invasion via an upregulation of BIN3 plus an EGF-induced tyrosine phosphorylation of DOCK7 on Y1118 (Fig. 1P, Fig. 2Q-T). The EGF-induced tyrosine phosphorylation of DOCK7 facilitates its association with BIN3 leading to inhibition of DOCK7. BIN3 exerts its anti-invasion effect by binding to and inhibiting DOCK7. Mutation of this (Y1118) residue results in a loss of DOCK7 phosphorylation in response to EGF and a loss of DOCK7-BIN3 association (Fig. 2Q-T).

DOCK7 is constitutively active and activates Rho GTPases that drive invasion. Under basal conditions DOCK7 is constitutively active suggesting that the small levels of BIN3 available does not significantly suppress the activity of DOCK7. We also undertook an experiment to examine whether silencing BIN3 results in any impact on DOCK7 and Rho GTPase activity. We found that silencing BIN3 has no effect on DOCK7 or Rho GTPase activity or invasion (Fig. 2N-P, Fig. 1R-S). Thus further decreasing the low level of cellular BIN3 cannot further decrease a non-existent or a very low level BIN3-DOCK7 association and has no effect on Rho GTPase activity or invasion. These data provide an explanation for why silencing of BIN3 in GBM12 cells does not lead to increased invasion or shortened survival (Fig. 3O).

On the other hand, increase in BIN3 by EGF (and EGF-induced tyrosine phosphorylation of DOCK7) or overexpressing BIN3 leads to a substantial association with DOCK7, inhibition of DOCK7 and of Rho GTPase activity (Fig. 2I-K, M-P, Supplementary Fig. 4N-R). This is a gain of function property of upregulated BIN3. Thus, we propose that EGFR ligand is required upregulate BIN3 to an adequate level to execute its tumor suppressor activity.

Comment 6. In Figure 5A-C, tof should be applied in the presence of EGF. The proliferation rate of GBMs treated with ToF should be provided to evaluate its' capacity as a potential drug.

Response:



Tofacitinib plus EGF: This has been done. Fig. 5A has been revised to include the effect of EGF. Adding tofacitinib plus EGF together does not result in further upregulation of BIN3 compared to EGF alone or tofacitinib alone (Supplementary Fig. 5V). This is consistent with our findings that tofacitinib upregulates BIN3 by an upregulation of EGFR ligand (Fig. 5G). Additionally, addition of EGF plus tofacitinib does not increase invasiveness compared to either agent alone (Supplementary Fig. 5W).

Proliferation rate

The proliferation of GBM12, GBM9 and GBM6 in response to tofacitinib are shown in Fig. 5Q. Consistent with the role of tofacitinib in upregulating EGFR ligand, tofacitinib increases proliferation.

In response to reviewer comment, we also examined proliferation in tofacitinib treated mouse tumors and found that proliferation is increased (Supplementary Fig. 5X-Z). This is consistent with our model in which tofacitinib upregulates BIN3 and suppresses invasion via a ligand-mediated activation of the EGFR. Importantly, despite the increased proliferation, tofacitinib suppresses invasion and results in an improved prognosis in multiple *in vivo* models (Fig. 6A-N).

Comment 7. The author claimed that “All of these studies have used established GBM cell lines that are no longer considered to be a representative model of GBM, in part because they lose the EGFR amplification” how can you ensure that your GSCs don’t lose the EGFR amplification? I mean, how if GBM6 GBM12 lose EGFR amplification, at least part of amplification compared with the GBM species.

Response: We acknowledge the importance of this question. To address whether the EGFR amplification and level of EGFR expression in the Mayo PDX lines changes over time we undertook the following experiments.

The Mayo PDX model involves implantation of resected GBMs in mice where they grow as subcutaneous tumors¹. The tumors are harvested and cell cultures are generated for short term use. In those cases in which we stably transfect these cultures, they are in culture longer, for antibiotic selection and expansion of clones. To determine whether EGFR amplification is lost or EGFR levels change in these lines, we undertook the following experiments.

1. Since a key issue is whether EGFR amplification and EGFR levels are maintained in stably transfected PDX lines, we compared the EGFR level and EGFR copy number in GBM12 and GBM6 cells stably transfected with TGF α and BIN3 (Supplementary Fig 4B-C). We compared the EGFR levels in freshly harvested cells to cells stably transfected with empty vector, TGF α or BIN3. EGFR levels are quite similar (Supplementary Fig. 4B-C). Taqman qPCR was done to detect EGFR amplification²⁻⁷

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and demonstrated no loss of EGFR copy number in stably transfected cells (Supplementary Fig. 4C).

2. We also compared the EGFR levels in orthotopic tumors. We compared tumors generated by injecting fresh GBM12 (or GBM6) cultures to tumors formed by injecting stably transfected Mayo PDX GBM12-TGF α or GBM12 BIN3 cultures. We found that EGFR levels in these tumors are quite similar (Supplementary Fig. 4L) This is also true for EGFR copy number (Supplementary Fig. 4M) demonstrating that the EGFR amplification is retained in tumors derived from stably transfected lines compared to tumors generated from fresh cultures.

3. Finally, Mayo PDX lines were cultured in serum containing medium for 0-8 weeks. We compared the level of EGFR expression by Western blot and EGFR copy number by Taqman qPCR. We found that EGFR level in cultures that have been in serum containing medium for 8 weeks are similar compared to EGFR levels from freshly harvested tumor cultures, when tested by Western blot (Supplementary Fig. 4D). When we looked at EGFR copy number, again we found very little change (Supplementary Fig. 4E). Also, after being cultured for eight weeks in serum containing medium, the cells continue to respond to EGFR stimulation with suppression of invasion, consistent with the stable EGFR levels Supplementary Fig. 4F

The experiments outlined above indicate that the EGFR levels in Mayo PDX cultures remain stable for the duration of our experiments. It is possible that the increased stability of EGFR in this cell culture model results from the initial passage in mice. Alternatively, and perhaps more likely, the duration of culture in serum containing medium is important. Previous studies⁸ distinguish between short term cultures (less than 90 days) and long term cultures (greater than 90 days), and suggest that the EGFR amplification, at least in some lines, is lost in long term cultures.

Our stable transfection and expansion of cultures generally take less than 2 months. Our new data indicate that the EGFR amplification is retained in the stably transfected Mayo PDX lines, and in the tumors that are derived from stably transfected Mayo PDX lines.

It should be noted that in this study we have also used a neurosphere model that is never cultured in serum. Almost every experiment in our paper is also conducted on primary GBM cultures maintained as neurospheres and cultured in stem cell medium and are known to more closely mirror the phenotype and genotype of primary tumors including the EGFR amplification⁹.

Our results in Mayo PDXs and in neurospheres are very similar.



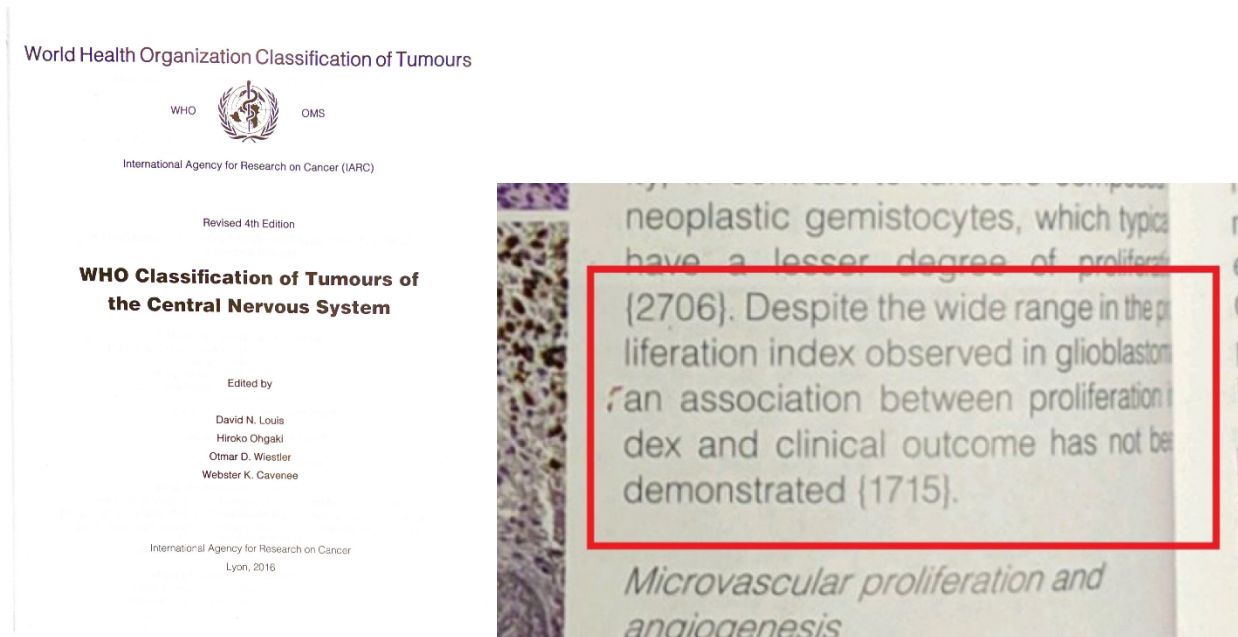
Comment 8. The title was not appropriate as the authors claimed that treated with EGF in constitute EGFR cells promotes the proliferation. This was also an oncogene character.

Response: We acknowledge that both proliferation and invasion are hallmarks of cancer. One of the surprising and provocative findings of our study is that in experimental models of GBM, invasion is required for tumor expansion. Thus, proliferation alone is not sufficient for tumor expansion. In the presence of EGFR ligand, invasion is suppressed, and proliferation is enhanced, but the net result is inhibition of tumor growth. The TCGA analysis also demonstrates that in EGFR amplified GBMs a low level of EGFR ligand is tumor suppressive while a high level of EGFR ligand is tumor suppressive (Fig. 7C). Thus, both our experimental findings and TCGA data indicate that EGFR ligand shifts the role of EGFR from oncogene to tumor suppressor (even though EGFR ligand increases proliferation, the suppression of invasion is the key determinant of the biological outcome). This is also quite consistent with TCGA analysis showing that high EGFR ligand in EGFR amplified GBMs confers a better prognosis compared to low ligand (Fig. 7D). The experimental data are shown in Figure 3 and Supplemental Figure 4.

Thus, ligand-activated EGFR signaling in EGFR amplified GBMs or high EGFR expressing cells both increases proliferation and suppresses invasion with the net result being inhibition of tumor expansion and smaller tumors. *We consider this a novel and important finding of this study.*

It is important to note that evidence demonstrating an independent prognostic role for Ki-67 or MIB1 (a marker of proliferation in GBM) has been elusive in GBM, at least in some studies¹⁸. We refer Reviewer 3 to the WHO classification of brain tumors (2016), the authoritative reference for neuropathologists. This text also comments on the lack of a correlation between Ki-67 and MIB-1 and cites the same reference as we have¹⁸. We have inserted the paragraph of the relevant page here.





We propose that this is a new biological insight from our study—that invasion is required for tumor expansion and that proliferation alone is not sufficient.

Comment 9. The author claimed that GBM6 was WT+VIII, WT was almost undetectable in Figure 1A. But extremely strong expressed in Figure 1D. Please explain. Uncut raw data should be provided.

Response: GBM6 expresses a high level of EGFRvIII and a small amount of EGFRwt. EGFRvIII is a truncated EGFR mutant that is missing exons 2-7 and migrates lower on an electrophoresis gel. We have now labeled EGFRwt and EGFRvIII in the Figure. GBM6 expresses a high level of EGFRvIII and a low level of EGFRwt. The strong band that shows up in Fig. 1D is EGFRvIII. We have repeated the gel (Fig. 1D) and run it longer to demonstrate both EGFRwt and EGFRvIII expression. We have also provided the uncut raw data.

Minor :

Comment 10. In Figure 1H, cells were transfected with siEGFR, but the immunoblot of EGFR

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indicated that EGFR was not knocked down. IB should be re applied to separate EGFR and EGFRVIII.

Response: In Fig. 1H, we show a rescue experiment. 3' UTR targeted EGFR siRNA was used followed by overexpression of EGFRwt or EGFRvIII. The overexpression of the EGFR rescues the siRNA knockdown of EGFR.

The gel was rerun to demonstrate separation of EGFRwt and EGFRvIII in GBM6.

Comment 11. In Figure 3C, the slice was not stained with HE. Please check.

Response: In Fig. 3C, the upper panel is stained with H&E and the lower panels are stained with SMI-31

Comment 12. Figure 3 should be moved to the supplementary figure and the data about EGF in vivo should be moved to the main Figure.

Response: We have moved the EGF in vivo data to main Fig. 3I-K. Part of Fig. 3 has been moved to Supplementary Fig. 4. Since there is key animal data showing the effect of EGFR ligand in Fig. 3, we have retained most of the data in Fig. 3 (but see below).

Comment 13. There were typos such as TFG and GCS. please check and correct.

Response: We corrected TFG and GCS. We also corrected other typos.

Comment 14 Figure 3J-M should be moved to the supplementary figure.

Response: Done. We have moved Fig. 3J-M to Supplementary Fig. 4N-R.

Comment 15. Figure 3M, 3P was mislabeled.

Response: We have corrected the mislabeling.

Comment 16. How do the authors identify NT region in GBM12B HE slice in Figure 3R?

Response: In normal tissue (NT), the cell density is low and the nuclei are small and lack cytological atypia.

Comment 17. In Figure 4A, IB should be re-applied to clearly separate EGFR and VIII.



Response: done

Comment 18. Figure 4I, 4H need to be re-applied in the presence of EGF.

Response: 4H repeat is done with EGF
4I repeat is done with EGF (now 4I-J).

Comment 19. There were some contradictions in Figure 4 D, 4I, 4L. knockdown of EMP1 and TAB1 rarely affected the invasion, please explain.

Response:

We suggest that TAB1 and EMP1 activity are required for EGFR mediated invasion but not sufficient by themselves to drive invasion

We are dealing with a complex biological system. We have identified a core pathway involving TAB1 and EMP1 that drives invasion, here but there are likely additional relevant EGFR driven signals that future work may uncover.

Comment 20. In Figure 4J, TAB1 decreased in GBM12 but increased in GBM9. Please explain. IB need to be redone to separate EGFR and EGFRvIII.

Response: We have rerun the EGFR gel to separate EGFRwt and EGFRvIII (now Fig. 4K).

Densitometry reveals a very small decrease in TAB1 when EGFR is overexpressed in GBM12 cells. In GBM9 cells overexpression of the EGFR results in a very small increase in TAB1. At this time, we are unsure of the significance or mechanism of these small changes.

Comment 21. The time point should be provided in Figure 5F as it was contradictory to the results in Figure 1I.

Response: In Fig. 5F we use cetuximab. In Fig. 1I we use erlotinib. The time point is 24h incubation with tofacitinib and cetuximab. It should be noted that cetuximab affects ligand binding, while erlotinib blocks the tyrosine kinase activity of the EGFR. Thus, they have distinct effects. For Fig. 5F we used cetuximab an antibody that blocks ligand binding to the EGFR and thus block ligand-dependent EGFR signaling. However, cetuximab would not block constitutive or ligand-independent EGFR signaling. Since invasion is driven by constitutive EGFR signaling, cetuximab fails to block it (Fig. 5F, Supplementary Fig. 1P-S). In Figure 1I we use erlotinib, an EGFR tyrosine kinase inhibitor. Both the constitutive and ligand-dependent EGFR signaling

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require the kinase activity of the EGFR and are blocked by erlotinib and thus the basal invasion is reduced by erlotinib (Fig. 1I).

Comment 22. What's the advantage of Tof? EGF was reported to inhibit the invasion as you mentioned previously. Why don't use EGF immediately?

Response:

Tofactinib crosses the blood brain barrier²⁶⁻²⁸, and is available in an oral form for rheumatologic conditions. EGF does not cross the blood brain barrier²⁹ and would have to be infused into the brain, a cumbersome procedure.

Comment 23. How do you suggest to culture GSCs as additional EGF will inhibit invasion?

Response: GSCs are cultured as non-adherent neurospheres in stem cell medium containing EGF and bFGF. There is no difficulty in culturing neurospheres since these cells are non-adherent, grow as suspension cultures and do not need to attach or invade. EGF stimulates proliferation in such cells.

Comment 24. The length of the article should be reduced to meet Nature cell biology demands. The data quality should be improved especially the IB assay and the raw data should be provided as well.

Response: Manuscript has been shortened.

Many immunoblots were redone to improve quality and to separate EGFRwt and EGFRvIII (give examples). Examples are, Fig. 1D, 1F, 1H, 4A, 4J and 4H. This is in addition to a number of new experiments and data.

Raw data and Uncropped blots are provided.



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Decision Letter, first revision:

Subject: Decision on Nature Cell Biology submission NCB-H46222A
Message: *Please delete the link to your author homepage if you wish to forward this email to co-authors.

Dear Dr Habib,

Your manuscript, "EGFR ligand shifts the role of EGFR from oncogene to tumor suppressor in EGFR amplified glioblastoma", has now been seen by the original referees. As you will see from their comments (attached below) they find this work of interest, but have raised some important points. Although we are also very interested in this study, we believe that their concerns should be addressed before we can consider publication in Nature Cell Biology. Please kindly note that this will be the last-round revision, and the reviewers must be fully satisfied before further consideration of the manuscript.

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Nature Cell Biology editors discuss the referee reports in detail within the editorial team, including the chief editor, to identify key referee points that should be addressed with priority, and requests that are overruled as being beyond the scope of the current study. To guide the scope of the revisions, I have listed these points below. We are committed to providing a fair and constructive peer-review process, so please feel free to contact me if you would like to discuss any of the referee comments further.

In particular, it would be essential to:

A) Address the persisting concerns from Reviewer 3;

B) Finally please pay close attention to our guidelines on statistical and methodological reporting (listed below) as failure to do so may delay the reconsideration of the revised manuscript. In particular please provide:

- a Supplementary Figure including unprocessed images of all gels/blots in the form of a multi-page pdf file. Please ensure that blots/gels are labeled and the sections presented in the figures are clearly indicated.

- a Supplementary Table including all numerical source data in Excel format, with data for different figures provided as different sheets within a single Excel file. The file should include source data giving rise to graphical representations and statistical descriptions in the paper and for all instances where the figures present representative experiments of multiple independent repeats, the source data of all repeats should be provided.

We therefore invite you to take these points into account when revising the manuscript. In addition, when preparing the revision please:

- ensure that it conforms to our format instructions and publication policies (see below and <https://www.nature.com/nature/for-authors>).

- provide a point-by-point rebuttal to the full referee reports verbatim, as provided at the end of this letter.

- provide the completed Reporting Summary (found here <https://www.nature.com/documents/nr-reporting-summary.pdf>). This is essential for reconsideration of the manuscript and will be available to



editors and referees in the event of peer review. For more information see <http://www.nature.com/authors/policies/availability.html> or contact me.

When submitting the revised version of your manuscript, please pay close attention to our [href="https://www.nature.com/nature-research/editorial-policies/image-integrity">Digital Image Integrity Guidelines](https://www.nature.com/nature-research/editorial-policies/image-integrity). and to the following points below:

- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
- that control panels for gels and western blots are appropriately described as loading on sample processing controls
- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

Nature Cell Biology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as ‘corresponding author’ on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on ‘Modify my Springer Nature account’. For more information please visit www.springernature.com/orcid.

This journal strongly supports public availability of data. Please place the data used in your paper into a public data repository, or alternatively, present the data as Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. Please note that for some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories appears below.

Please submit the revised manuscript files and the point-by-point rebuttal to the referee comments using this link:



[REDACTED]

*This url links to your confidential home page and associated information about manuscripts you may have submitted or be reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We would like to receive the revision within four weeks. If submitted within this time period, reconsideration of the revised manuscript will not be affected by related studies published elsewhere, or accepted for publication in Nature Cell Biology in the meantime. We would be happy to consider a revision even after this timeframe, but in that case we will consider the published literature at the time of resubmission when assessing the file.

We hope that you will find our referees' comments, and editorial guidance helpful. Please do not hesitate to contact me if there is anything you would like to discuss.

Best wishes,
Zhe Wang

Zhe Wang, PhD
Senior Editor
Nature Cell Biology

Tel: +44 (0) 207 843 4924
email: zhe.wang@nature.com

Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

The authors have extensively revised the original version of the manuscript and carried out a significant number of new experiments. I believe they have appropriately addressed all the issues raised in my original review, and I am happy to recommend publication of the revised version.

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Reviewer #2:

Remarks to the Author:

Revised manuscript adequately addresses issues raised in prior review.

Reviewer #3:

Remarks to the Author:

General comment: in the revision of “EGFR ligand shifts the role of EGFR from oncogene to tumor suppressor in EGFR amplified glioblastoma”. Most issues were addressed but some are still there.

Comment 1(related to response 1): I accept that “constitutive signaling is defined as signaling triggered by EGFRwt or EGFRvIII expression leading to spontaneous dimerization and downstream signaling in the absence of EGFR ligand”. In the paper the author previous published, constitutive signaling is due to the overexpression of EGFR wt or EGFRvIII. The presence of EGFRvIII represents the constitutive signaling while the EGFR wt can't.

Cells shifts ligand dependent EGFR signaling to ligand-independent EGFR signaling by overexpressing EGFRwt or EGFRvIII, which was fully demonstrated in the paper. However, the authors still don't answer whether ligand independent signaling can shift to ligand dependent signaling by reducing the expression of EGFR. In the paper, the knock down of EGFR totally inhibits the EGFR wt while the remaining EGFRvIII apparently don't bind with EGF. This was too opportunistic, and the following experiments were needed.

1. Knock EGFR down in GBM 12 which only contain EGFRwt in a dosage dependent manner. It was reachable because in Figure1D 1H, different level of EGFR was accessible.
2. Detect the pEGFR and downstream signaling without any ligands in the cells above to decide it's ligand dependent/independent.
3. Detect the invasion percentage in the cells treated with EGF.
4. Detect the TAB1-BIN3 axes as well.

Comment 2(related to response 2): The authors suggested the level of pEGFR plays key role in inducing different phenotype. However, quantitative statistics were needed. In recently published paper, “Glioblastoma mutations alter EGFR dimer structure to prevent ligand bias”, EGFR reacts to different ligands and induce different downstream cascade because the extracellular mutations and the transcellular structure varies. It's more acceptable than just comparing the level of p-EGFR.

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Comment 3(related to response 3): U251 was obviously a ligand induced EGFR signaling because the authors overexpress EGFR and EGFRvIII and treat them with or without EGF. The gene background I mentioned here was the cells with ligand dependent EGFR signaling was totally different from cells with ligand-independent signaling. It's unconvincing to study ligand independent signaling with the background of ligand dependent signaling. EGFR signaling was not an isolated pathway, the interaction may vary across cells. Moreover, although the dysregulation of BIN3 was confirmed in GSCs, it may not be the major reason. The transcriptome between GSCs and U251 varies. The RNA seq in GSC was still needed and before that, TAB1-BIN3 axis was still unconvincing.

GUIDELINES FOR SUBMISSION OF NATURE CELL BIOLOGY ARTICLES

READABILITY OF MANUSCRIPTS – Nature Cell Biology is read by cell biologists from diverse backgrounds, many of whom are not native English speakers. Authors should aim to communicate their findings clearly, explaining technical jargon that might be unfamiliar to non-specialists, and avoiding non-standard abbreviations. Titles and abstracts should concisely communicate the main findings of the study, and the background, rationale, results and conclusions should be clearly explained in the manuscript in a manner accessible to a broad cell biology audience. Nature Cell Biology uses British spelling.

ARTICLE FORMAT

TITLE – should be no more than 100 characters including spaces, without punctuation and avoiding technical terms, abbreviations, and active verbs..

AUTHOR NAMES – should be given in full.



AUTHOR AFFILIATIONS – should be denoted with numerical superscripts (not symbols) preceding the names. Full addresses should be included, with US states in full and providing zip/post codes. The corresponding author is denoted by: "Correspondence should be addressed to [initials]."

ABSTRACT – should not exceed 150 words and should be unreferenced. This paragraph is the most visible part of the paper and should briefly outline the background and rationale for the work, and accurately summarize the main results and conclusions. Key genes, proteins and organisms should be specified to ensure discoverability of the paper in online searches.

TEXT – the main text consists of the Introduction, Results, and Discussion sections and must not exceed 3500 words including the abstract. The Introduction should expand on the background relating to the work. The Results should be divided in subsections with subheadings, and should provide a concise and accurate description of the experimental findings. The Discussion should expand on the findings and their implications. All relevant primary literature should be cited, in particular when discussing the background and specific findings.

ACKNOWLEDGEMENTS – should be kept brief. Professional titles and affiliations are unnecessary. Grant numbers can be listed.

AUTHOR CONTRIBUTIONS – must be included after the Acknowledgements, detailing the contributions of each author to the paper (e.g. experimental work, project planning, data analysis etc.). Each author should be listed by his/her initials.

FINANCIAL AND NON-FINANCIAL COMPETING INTERESTS – the authors must include one of three declarations: (1) that they have no financial and non-financial competing interests; (2) that they have financial and non-financial competing interests; or (3) that they decline to respond, after the Author Contributions section. This statement will be published with the article, and in cases where financial and non-financial competing interests are declared, these will be itemized in a web supplement to the article. For further details please see <https://www.nature.com/licenceforms/nrg/competing-interests.pdf>.

REFERENCES – are limited to a total of 70 in the main text and Methods combined. They must be numbered sequentially as they appear in the main text, tables and figure legends and Methods and must follow the precise style of Nature Cell Biology references. References only cited in the Methods should be numbered consecutively following the last reference cited in the main text. References only associated with Supplementary Information (e.g. in supplementary legends) do not count toward the



total reference limit and do not need to be cited in numerical continuity with references in the main text. Only published papers can be cited, and each publication cited should be included in the numbered reference list, which should include the manuscript titles. Footnotes are not permitted.

METHODS – Nature Cell Biology publishes methods online. The methods section should be provided as a separate Word document, which will be copyedited and appended to the manuscript PDF, and incorporated within the HTML format of the paper.

Methods should be written concisely, but should contain all elements necessary to allow interpretation and replication of the results. As a guideline, Methods sections typically do not exceed 3,000 words. The Methods should be divided into subsections listing reagents and techniques. When citing previous methods, accurate references should be provided and any alterations should be noted. Information must be provided about: antibody dilutions, company names, catalogue numbers and clone numbers for monoclonal antibodies; sequences of RNAi and cDNA probes/primers or company names and catalogue numbers if reagents are commercial; cell line names, sources and information on cell line identity and authentication. Animal studies and experiments involving human subjects must be reported in detail, identifying the committees approving the protocols. For studies involving human subjects/samples, a statement must be included confirming that informed consent was obtained. Statistical analyses and information on the reproducibility of experimental results should be provided in a section titled “Statistics and Reproducibility”.

All Nature Cell Biology manuscripts submitted on or after March 21 2016, must include a Data availability statement as a separate section after Methods but before references, under the heading “Data Availability”. For Springer Nature policies on data availability see <http://www.nature.com/authors/policies/availability.html>; for more information on this particular policy see <http://www.nature.com/authors/policies/data/data-availability-statements-data-citations.pdf>. The Data availability statement should include:

- Accession codes for primary datasets (generated during the study under consideration and designated as “primary accessions”) and secondary datasets (published datasets reanalysed during the study under consideration, designated as “referenced accessions”). For primary accessions data should be made public to coincide with publication of the manuscript. A list of data types for which submission to community-endorsed public repositories is mandated (including sequence, structure, microarray, deep sequencing data) can be found here <http://www.nature.com/authors/policies/availability.html#data>.



- Unique identifiers (accession codes, DOIs or other unique persistent identifier) and hyperlinks for datasets deposited in an approved repository, but for which data deposition is not mandated (see here for details <http://www.nature.com/sdata/data-policies/repositories>).

- At a minimum, please include a statement confirming that all relevant data are available from the authors, and/or are included with the manuscript (e.g. as source data or supplementary information), listing which data are included (e.g. by figure panels and data types) and mentioning any restrictions on availability.

- If a dataset has a Digital Object Identifier (DOI) as its unique identifier, we strongly encourage including this in the Reference list and citing the dataset in the Methods.

We recommend that you upload the step-by-step protocols used in this manuscript to the Protocol Exchange. More details can found at www.nature.com/protocolexchange/about.

DISPLAY ITEMS – main display items are limited to 6-8 main figures and/or main tables. For Supplementary Information see below.

FIGURES – Colour figure publication costs \$395 per colour figure. All panels of a multi-panel figure must be logically connected and arranged as they would appear in the final version. Unnecessary figures and figure panels should be avoided (e.g. data presented in small tables could be stated briefly in the text instead).

All imaging data should be accompanied by scale bars, which should be defined in the legend. Cropped images of gels/blots are acceptable, but need to be accompanied by size markers, and to retain visible background signal within the linear range (i.e. should not be saturated). The boundaries of panels with low background have to be demarked with black lines. Splicing of panels should only be considered if unavoidable, and must be clearly marked on the figure, and noted in the legend with a statement on whether the samples were obtained and processed simultaneously. Quantitative comparisons between samples on different gels/blots are discouraged; if this is unavoidable, it has to be performed for samples derived from the same experiment with gels/blots were processed in parallel, which needs to be stated in the legend.

Figures should be provided at approximately the size that they are to be printed at (single column is 86 mm, double column is 170 mm) and should not exceed an A4 page (8.5 x 11"). Reduction to the scale



that will be used on the page is not necessary, but multi-panel figures should be sized so that the whole figure can be reduced by the same amount at the smallest size at which essential details in each panel are visible. In the interest of our colour-blind readers we ask that you avoid using red and green for contrast in figures. Replacing red with magenta and green with turquoise are two possible colour-safe alternatives. Lines with widths of less than 1 point should be avoided. Sans serif typefaces, such as Helvetica (preferred) or Arial should be used. All text that forms part of a figure should be rewritable and removable.

We accept files from the following graphics packages in either PC or Macintosh format:

- For line art, graphs, charts and schematics we prefer Adobe Illustrator (.AI), Encapsulated PostScript (.EPS) or Portable Document Format (.PDF). Files should be saved or exported as such directly from the application in which they were made, to allow us to restyle them according to our journal house style.

- We accept PowerPoint (.PPT) files if they are fully editable. However, please refrain from adding PowerPoint graphical effects to objects, as this results in them outputting poor quality raster art. Text used for PowerPoint figures should be Helvetica (preferred) or Arial.

- We do not recommend using Adobe Photoshop for designing figures, but we can accept Photoshop generated (.PSD or .TIFF) files only if each element included in the figure (text, labels, pictures, graphs, arrows and scale bars) are on separate layers. All text should be editable in 'type layers' and line-art such as graphs and other simple schematics should be preserved and embedded within 'vector smart objects' - not flattened raster/bitmap graphics.

- Some programs can generate Postscript by 'printing to file' (found in the Print dialogue). If using an application not listed above, save the file in PostScript format or email our Art Editor, Allen Beattie for advice (a.beattie@nature.com).

Regardless of format, all figures must be vector graphic compatible files, not supplied in a flattened raster/bitmap graphics format, but should be fully editable, allowing us to highlight/copy/paste all text and move individual parts of the figures (i.e. arrows, lines, x and y axes, graphs, tick marks, scale bars etc). The only parts of the figure that should be in pixel raster/bitmap format are photographic images or 3D rendered graphics/complex technical illustrations.

All placed images (i.e. a photo incorporated into a figure) should be on a separate layer and independent from any superimposed scale bars or text. Individual photographic images must be a minimum of 300+



DPI (at actual size) or kept constant from the original picture acquisition and not decreased in resolution post image acquisition. All colour artwork should be RGB format.

FIGURE LEGENDS – must not exceed 350 words for each figure to allow fit on a single printed NCB page together with the figure. They must include a brief title for the whole figure, and short descriptions of each panel with definitions of the symbols used, but without detailing methodology.

TABLES – main tables should be provided as individual Word files, together with a brief title and legend. For supplementary tables see below.

SUPPLEMENTARY INFORMATION – Supplementary information is material directly relevant to the conclusion of a paper, but which cannot be included in the printed version in order to keep the manuscript concise and accessible to the general reader. Supplementary information is an integral part of a Nature Cell Biology publication and should be prepared and presented with as much care as the main display item, but it must not include non-essential data or text, which may be removed at the editor's discretion. All supplementary material is fully peer-reviewed and published online as part of the HTML version of the manuscript. Supplementary Figures and Supplementary Notes are appended at the end of the main PDF of the published manuscript.

Supplementary items should relate to a main text figure, wherever possible, and should be mentioned sequentially in the main manuscript, designated as Supplementary Figure, Table, Video, or Note, and numbered continuously (e.g. Supplementary Figure 1, Supplementary Figure 2, Supplementary Table 1, Supplementary Table 2 etc.).

Unprocessed scans of all key data generated through electrophoretic separation techniques need to be presented in a supplementary figure that should be labeled and numbered as the final supplementary figure, and should be mentioned in every relevant figure legend. This figure does not count towards the total number of figures and is the only figure that can be displayed over multiple pages, but should be provided as a single file, in PDF or TIFF format. Data in this figure can be displayed in a relatively informal style, but size markers and the figures panels corresponding to the presented data must be indicated.

The total number of Supplementary Figures (not including the “unprocessed scans” Supplementary Figure) should not exceed the number of main display items (figures and/or tables (see our Guide to Authors and March 2012 editorial <http://www.nature.com/ncb/authors/submit/index.html#suppinfo>;



<http://www.nature.com/ncb/journal/v14/n3/index.html#ed>). No restrictions apply to Supplementary Tables or Videos, but we advise authors to be selective in including supplemental data.

Each Supplementary Figure should be provided as a single page and as an individual file in one of our accepted figure formats and should be presented according to our figure guidelines (see above). Supplementary Tables should be provided as individual Excel files. Supplementary Videos should be provided as .avi or .mov files up to 50 MB in size. Supplementary Figures, Tables and Videos must be accompanied by a separate Word document including titles and legends.

GUIDELINES FOR EXPERIMENTAL AND STATISTICAL REPORTING

REPORTING REQUIREMENTS – We ask authors to complete a Reporting Summary that collects information on experimental design and reagents. We hope this will aid in your evaluation of the paper. The Reporting Summary can be found here <https://www.nature.com/documents/nr-reporting-summary.pdf>) Please note that these forms are dynamic ‘smart pdfs’ and must therefore be downloaded and completed in Adobe Reader. We will then flatten them for ease of use. If you would like to reference the guidance text as you complete the template, please access these flattened versions at <http://www.nature.com/authors/policies/availability.html>.

STATISTICS – Wherever statistics have been derived the legend needs to provide the n number (i.e. the sample size used to derive statistics) as a precise value (not a range), and define what this value represents. Error bars need to be defined in the legends (e.g. SD, SEM) together with a measure of centre (e.g. mean, median). Box plots need to be defined in terms of minima, maxima, centre, and percentiles. Ranges are more appropriate than standard errors for small data sets. Wherever statistical significance has been derived, precise p values need to be provided and the statistical test used needs to be stated in the legend. Statistics such as error bars must not be derived from $n < 3$. For sample sizes of $n < 5$ please plot the individual data points rather than providing bar graphs. Deriving statistics from technical replicate samples, rather than biological replicates is strongly discouraged. Wherever statistical significance has been derived, precise p values need to be provided and the statistical test stated in the legend.

Information on how many times each experiment was repeated independently with similar results needs to be provided in the legends and/or Methods for all experiments, and in particular wherever representative experiments are shown.



We strongly recommend the presentation of source data for graphical and statistical analyses as a separate Supplementary Table, and request that source data for all independent repeats are provided when representative experiments of multiple independent repeats, or averages of two independent experiments are presented. This supplementary table should be in Excel format, with data for different figures provided as different sheets within a single Excel file. It should be labelled and numbered as one of the supplementary tables, titled “Statistics Source Data”, and mentioned in all relevant figure legends.

----- Please don't hesitate to contact NCB@nature.com should you have queries about any of the above requirements -----

Author Rebuttal, first revision:

Response to Reviewer Comments

Reviewer 1

Comment: The authors have extensively revised the original version of the manuscript and carried out a significant number of new experiments. I believe they have appropriately addressed all the issues raised in my original review, and I am happy to recommend publication of the revised version.

Response: Thank you.

Reviewer 2

Comment: Revised manuscript adequately addresses issues raised in prior review.

Response: Thank you.

Reviewer 3

General comment : in the revision of “EGFR ligand shifts the role of EGFR from oncogene to tumor suppressor in EGFR amplified glioblastoma”. Most issues were addressed but some are still there.

Response:

We thank Reviewer 3 for a thoughtful and detailed critique that has helped to strengthen our manuscript. We appreciate the Reviewer’s comment that most issues were addressed. We have now

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addressed all remaining issues as recommended by Reviewer 3.

Comment 1(related to response 1): I accept that “constitutive signaling is defined as signaling triggered by EGFRwt or EGFRvIII expression leading to spontaneous dimerization and downstream signaling in the absence of EGFR ligand”. In the paper the author previous published, constitutive signaling is due to the overexpression of EGFR wt or EGFRvIII. The presence of EGFRvIII represents the constitutive signaling while the EGFR wt can’t.

Cells shifts ligand dependent EGFR signaling to ligand-independent EGFR signaling by overexpressing EGFRwt or EGFRvIII, which was fully demonstrated in the paper. However, the authors still don’t answer whether ligand independent signaling can shift to ligand dependent signaling by reducing the expression of EGFR. In the paper, the knock down of EGFR totally inhibits the EGFR wt while the remaining EGFRvIII apparently don’t bind with EGF. This was too opportunistic, and the following experiments were needed.

1. Knock EGFR down in GBM 12 which only contain EGFRwt in a dosage dependent manner. It was reachable because in Figure1D 1H, different level of EGFR was accessible.
2. Detect the pEGFR and downstream signaling without any ligands in the cells above to decide it’s ligand dependent/independent.
3. Detect the invasion percentage in the cells treated with EGF.
4. Detect the TAB1-BIN3 axes as well.

Response:

We performed the experiments as suggested by Reviewer 3.

We found that GBM12 transfected with different doses of EGFR siRNA shows a dose-dependent decrease in EGFR protein (Extended DataFig. 2h-i).

An invasion assay demonstrates that EGF still suppresses invasion of GBM12 with 5 pmol of siRNA, while EGF increases invasion of GBM12 with 20 or 50 pmol of siRNA , indicating that as predicted by Reviewer 3 , invasion that was previously driven by ligand-independent signaling is now driven by ligand-dependent signaling with reduced EGFR levels (Extended Data Fig. 2h-i).

We also investigated pEGFR and downstream signaling events including the TAB-1 and BIN3 signaling axis at different levels of EGFR expression without any ligands. We found that reducing EGFR level results in decreased levels of pEGFR, pTAK1, pp65 and EMP1 protein. However there is no effect on the levels of BIN3, EGR1, pDOCK7 and DOCK7 (Extended Data Fig. 2h). These data support our other data distinguishing between the ligand-independent and ligand-dependent signals.



Comment 2 (related to response 2): The authors suggested the level of pEGFR plays key role in inducing different phenotype. However, quantitative statistics were needed. In recently published paper, “Glioblastoma mutations alter EGFR dimer structure to prevent ligand bias”, EGFR reacts to different ligands and induce different downstream cascade because the extracellular mutations and the transcellular structure varies. It’s more acceptable than just comparing the level of p-EGFR.

Response (add information):

We have done quantitative statistics by performing densitometry using Image J for Extended Data Fig. 1t and for Extended Data Fig. 5n as shown in Extended Data Fig. 1u and Extended Data Fig. 5o and as described in the figure legends. We have added a citation for the recently published paper mentioned by Reviewer 3 in our manuscript

Comment 3 (related to response 3): U251 was obviously a ligand induced EGFR signaling because the authors overexpress EGFR and EGFRvIII and treat them with or without EGF. The gene background I mentioned here was the cells with ligand dependent EGFR signaling was totally different from cells with ligand-independent signaling. It’s unconvincing to study ligand independent signaling with the background of ligand dependent signaling. EGFR signaling was not an isolated pathway, the interaction may vary across cells. Moreover, although the dysregulation of BIN3 was confirmed in GSCs, it may not be the major reason. The transcriptome between GSCs and U251 varies. The RNA seq in GSC was still needed and before that, TAB1-BIN3 axis was still unconvincing.

(Reviewer 3 had also commented in the previous critique: The microarray you used in ref 2 was not suitable here. You compare EGFR-low cells with EGFR-low + EGFR cells. This was not correct because the genetic background of EGFR-low cells and EGFR amplified cells were different. In this paper, you should collect GBMs (GBM6 GBM12) treated with or without EGF and subjected to RNA sequence. Thus, the selection of BIN3 was unconvincing. Moreover, the microarray data was applied in U251MG, which was not acceptable in this paper, GSC should be applied instead).

Response: We acknowledge that Reviewer 3’s argument is correct. As recommended by Reviewer 3 we undertook RNA seq in GBM12 with or without EGF. The RNA seq experiment confirms a significant upregulation of BIN3 by EGF in GBM12 cells (Extended Data Fig. 2r). We have deposited the RNA seq data in Sequence Read Archive (SRA) with accession number PRJNA812870. A robust upregulation of BIN3 by EGF is noted at an mRNA and protein level for multiple PDX lines GBM36 (Fig. 1p-q).



Decision Letter, second revision:

Subject: Your manuscript, NCB-H46222B

Message:

Our ref: NCB-H46222B

25th March 2022

Dear Dr. Habib,

Thank you for submitting your revised manuscript "EGFR ligand shifts the role of EGFR from oncogene to tumor suppressor in EGFR amplified glioblastoma" (NCB-H46222B). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Cell Biology, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

If the current version of your manuscript is in a PDF format, please email us a copy of the file in an editable format (Microsoft Word or LaTeX)-- we can not proceed with PDFs at this stage.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Cell Biology Please do not hesitate to contact me if you have any questions.

Sincerely,

Zhe Wang, PhD
Senior Editor
Nature Cell Biology

Tel: +44 (0) 207 843 4924

email: zhe.wang@nature.com

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Reviewer #3 (Remarks to the Author):

The authors have addressed all my concerns in the previous round review. I am satisfied with the authors responses, and happy to recommoned pubilsihing this interesting study in Nature Cell Biology.

Decision Letter, final requests:

Subject: NCB: Your manuscript, NCB-H46222B
Message: Our ref: NCB-H46222B

18th April 2022

Dear Dr. Habib,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Cell Biology manuscript, "EGFR ligand shifts the role of EGFR from oncogene to tumor suppressor in EGFR amplified glioblastoma" (NCB-H46222B). Please carefully follow the step-by-step instructions provided in the attached file, and add a response in each row of the table to indicate the changes that you have made. Ensuring that each point is addressed will help to ensure that your revised manuscript can be swiftly handed over to our production team.

We would like to start working on your revised paper, with all of the requested files and forms, as soon as possible (preferably within one week). Please get in contact with us if you anticipate delays.

When you upload your final materials, please include a point-by-point response to any remaining reviewer comments.

If you have not done so already, please alert us to any related manuscripts from your group that are under consideration or in press at other journals, or are being written up for submission to other journals (see: <https://www.nature.com/nature-research/editorial-policies/plagiarism#policy-on-duplicate-publication> for details).

In recognition of the time and expertise our reviewers provide to Nature Cell Biology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "EGFR ligand shifts the role of EGFR from oncogene to tumor suppressor in EGFR

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amplified glioblastoma". For those reviewers who give their assent, we will be publishing their names alongside the published article.

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If you have any further questions, please feel free to contact us. Thank you!

Best regards,

Ziqian Li
Editorial Assistant
Nature Cell Biology

On behalf of

Zhe Wang, PhD
Senior Editor



Nature Cell Biology

Tel: +44 (0) 207 843 4924

email: zhe.wang@nature.com

Reviewer #3:

Remarks to the Author:

The authors have addressed all my concerns in the previous round review. I am satisfied with the authors' responses, and happy to recommend publishing this interesting study in Nature Cell Biology.

Final Decision Letter:

Subject: Decision on Nature Cell Biology submission NCB-H46222C

Message:

Dear Dr Habib,

I am pleased to inform you that your manuscript, "EGFR ligand shifts the role of EGFR from oncogene to tumor suppressor in EGFR amplified glioblastoma through BIN3 upregulation", has now been accepted for publication in Nature Cell Biology.

Thank you for sending us the final manuscript files to be processed for print and online production, and for returning the manuscript checklists and other forms. Your manuscript will now be passed to our production team who will be in contact with you if there are any questions with the production quality of supplied figures and text.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Cell Biology style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required.

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Please feel free to contact us if you have any questions.

With kind regards,

Zhe Wang, PhD
Senior Editor
Nature Cell Biology

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